Investigating the role of miRNA-mediated regulation in antipsychotic treatment response in a South African first-episode schizophrenia cohort

by
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March 2020

DECLARATION

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ABSTRACT

Although considerable advances in genomics research, with the likes of Genome-Wide Association Studies (GWAS), have uncovered vast quantities of genomic data regarding the genetic basis of many neuropsychiatric disorders, the heritability of such disorders and associated traits such as treatment response are not completely understood. Antipsychotic treatment response (ATR) of schizophrenia (SZ) is effective in only around half of all diagnosed patients. As with the disorder itself, ATR is known to be a complex trait with variable outcomes for patients and for which no clinical biomarkers exist to point towards possible indicators of treatment efficiency, often accompanied by the trial-and-error process of treatment adjustment. The large-scale nature of studies like GWAS, have the potential to elucidate this 'missing heritability' seen in ATR outcomes, in combination with pharmacogenetics approaches which aim to quantify on an individual level the interaction between genetics and maximal treatment efficacy, as well as in avoidance of potential adverse drug reactions (ADRs). The data outputs from GWAS have also highlighted noncoding regions of the genome, in which many top resultant hits have been shown to occur, in spite of this the interpretation of many genomic results have been limited to the nearest gene. While genetic variants linked to aspects of treatment like ATR and ADRs have been found, the interpretation of such results are important in discerning functional consequence surrounding identification of single nucleotide polymorphisms (SNPs) in association with either disorder or trait surrounding the disorder, *i.e.* ATR. These findings in combination with the knowledge of noncoding regions prominent role in hosting many SNPs, draws attention to the potential role of regulatory mechanisms interacting within ATR systems or pathways that have otherwise been disregarded. This study investigated the role of miRNA-mediated regulation in ATR, with parallel approaches designed to investigate the potential of miRNA implicated SNPs and miRNA-targeted genes in a bioinformatic systems genetics approach to reveal underlying regulatory consequences interacting in SZ ATR. The most significant finding identified a novel association of the variant, rs895808, with an improved treatment response for the negative symptom domain of the Positive and Negative Syndrome Scale (PANSS). The SNP was found to have consequential impact in disrupting the following miRNAs conserved sites; miRNA-548ac, miRNA-548d-3p, miRNA-548h-3p and miRNA-548z, and is linked to miRNA-4536 with unknown regulatory impact. FUMA analysis identified implicated pathways from miRNA-targeted genes and SNPs, with regulation of apoptosis and phosphodiesterase pathways presenting. Both pathways have largely been implicated in SZ pathophysiology, however this is the first identification with regard to miRNA-involvement to our knowledge and suggests an alternate avenue for investigation into the ATR of SZ.

OPSOMMING

Alhoewel aansienlik baie vordering in geonomiese navorsing, soos die van Genoomwye Assosiasiestudies (GWAS), tot groot hoeveelhede geonomiese data aangaande die genetiese basis van baie neuropsigiatriese toestande gelei het - word die oorerflikheid van sulke toestande en geassosieerde kenmerke soos behandelingsrespons nog nie volledig verstaan nie. Die antipsigotiese behandeling van skisofremie (SZ) is slegs effektief in min of meer die halfte van gediagnoseerde pasiente. Nes die toestand self is die antipsigotiese behandelingsrespons (ATR) bekend daarvoor om ‘n ingewikkelde eienskap te wees met veelvuldige moontlike uitkomste vir pasiente. Daar bestaan ook geen klieniese biomerkers om moontlike behandelingseffektiwiteit meer aan te dui nie, en behandeling gaan dikwels gepaard met steekproef proses van probeer en aanpas tot en met sukses behaal word. Die grootskaalse natuur van studies soos GWAS die potensiaal om hierdie ‘onbekende oorerflikheid’ wat in ATR gesien word te verklaar. In kombinasie daarmee help farmakogenetiese benaderings wat die interaksie tussen genetika en maksimum behandelingseffektiwitiet, asook vermyding van potensiële ongunstige medisyne reaksies (ADRs) om op ‘n indivduële vlak te teiken. Die dataopbrengste van GWAS het ook nie nie-koderende streke van die genoom uitgewys waarin baie van die mees gereëlde vangskote voorgekom het. Ten spyte van hiervan is die interpretasie van baie geonomiese uitkomstes beperk tot die naaste enkele geen. Terwyl genetiese variante gekoppel aan behandelingsaspekte soos ATR en ADRs al gevind is, is die interptretasie van hierdie data baie belangrik. Dit kan gebruik word om die funksionële nagevolge rondom die identifikasie van enkel nukleotied polimorfismes (SNPs) te onderskei, in genootskap met die toestand of ‘n kenmerk van die toestand - d.w.s ATR. Hierdie bevindinge, tesame met die kennis van die prominente rol wat nie-koderende streke speel in die huisvesting van SNPs vestig aandag op die potensiële rol van beherende meganismes wat op mekaar inwerk binne ATR sisteme of padweë wat andersins ter syde gesit sou word. Hierdie studie het die rol van miRNA-bemiddelde regulasie in ATR ondersoek, met paralelle benaderinge ontwerp om die potensiaal van miRNA geimpliseerd in SNPs en miRNA-geteikende gene in bioinformatiese sisteemsgenetieka te ondersoek om onderliggende beheersnagevolge wat met SZ ATR verkeer. Die mees betekenisvolle bevinding was ‘n nuwe assosiaise van die rs895808 variant, wat ‘n verbeterde behandelingsrespons vir negatiewe simptome op die Positiewe- en Negatiewe Sindroomskaal (PANSS). Dié SNP was gevind om gevolglike impak te om die gekonserfeerde streke van volgende miRNAs te versteur: miRNA-548ac, miRNA-548d-3p, miRNA-548h-3p en miRNA-548z. Dit is ook gekoppel aan miRNA-4536 met onbekende beherende impak. FUMA analise het padweë van geimpliseerde miRNA-geteikende gene geïdentifiseer, asook SNPs wat apoptose en fosodïesterase padweë reguleer. Beide padweë is grotendeels geïmpliseerd in SZ patofisiologie, alhoewel dit die eerste identifikasie met betrekking tot miRNA is en ‘n alternatiewe roete vir ondersoek van ATR van SZ is.

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LIST OF SYMBOLS AND ABBREVIATIONS

3’	3-prime end
%	Percentage
=	Equal to
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
Δ	Change in
©	Copyright
™	Trademark
ADRs	Adverse Drug Reactions/Responses
ADNP	Activity-dependent neuroprotective protein gene
Ago1-4	Argonaute protein 1-4
AIMs	Ancestry Informative Markers
APs	Antipsychotics
ARC	Activity-regulated cytoskeleton-associated protein gene
ASAP	Apoptosis and splicing associated protein complex
ASB16	Ankyrin repeat and SOCS box protein 16 gene
ASB16-AS1	Ankyrin repeat and SOCS box protein 16 antisense RNA 1 gene
ASD	Autism Spectrum Disorder
ATR	Antipsychotic Treatment Response
BD	Bipolar Disorder
cAMP	cyclic Mono Phosphate
CATIE	Clinical Antipsychotic Trials of Intervention Effects
cGMP	cyclic Guanosine Mono Phosphate
CNV	Copy number variant
COMT	Catechol-O-methyltransferase gene
CUtLASS	Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia
CYP	Cytochrome P450 enzyme family
CYP2D6	Cytochrome P450 2D6 enzyme gene
CYP2C9	Cytochrome P450 2C9 enzyme gene
DALYs	Disability-Adjusted Life-Years
DGCR8	DiGeorge syndrome chromosomal region 8 gene
DISC1	Disrupted in schizophrenia 1 gene
DNA	Deoxyribonucleic Acid
DNAJA3	DNAJ heat shock protein family member A3 gene
DNMT	DNA methyltransferase gene family
DRD1	Dopamine D_1 receptor gene
DRD2	Dopamine D_2 receptor gene
DRD3	Dopamine D_3 receptor gene
DRD4	Dopamine D_4 receptor gene
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, fourth edition
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, fifth edition
DTNBP1	Dysbindin gene
DUP	Duration of untreated psychosis

EJC	Exon junction complex
ENCODE	Encyclopedia of DNA Elements
EPS	Extrapyramidal Symptoms
EPSAE	Extrapyrimidal Adverse Events
eQTL	Expressive quantitative trait loci
FES	First-Episode Schizophrenia
FGA	First-Generation Antipsychotic
GAD	Glutamate decarboxylase gene
GRM2	Metabotropic glutamate 2 receptor gene
GWAS	Genome-Wide Association Studies
HDAC	Histone deacetylase gene family
HDAC2	Histone deacetylase gene 2
HDAC4	Histone deacetylase gene 4
HDAC5	Histone deacetylase gene 5
HERPUD1	Homocysteine inducible ER protein with ubiquitin like domain 1 gene
HIV	Human Immunodeficiency Virus
HTR2A	Serotonin receptor 2A gene
HWE	Hardy-Weinberg Equilibrium
H3k27me2/3	Lysine 27 in histone H3 complex
ICD-10	International Classification of Disease, version 10
iPSC	Induced pluripotent stem cell
Indel	Insertion and/or deletion
IL2	Interleukin 2 gene
KEGG	Kyoto Encyclopedia of Genes and Genomes
Kb	Kilobase
LAI	Long-acting injectable
LD	Linkage Disequilibrium
Lin-14	Heterochronic protein, lin-14
limk1	LIM domain kinase 1 mouse gene
lncRNA	Long noncoding RNA
MAF	Minor allele frequency
MAPK	Mitogen-activated Protein Kinase
Mb	Megabase
MEF2C	Myocyte specific enhancer factor 2C gene
MEF2D	Myocyte specific enhancer factor 2D gene
mGlu2	Metabotropic glutamate 2 receptor
MHC	Major Histocompatibility Complex
MIR137HG	MiRNA-137 gene
mRNA	Messenger RNA
NGR1	Neuroregulin 1 gene
NIHREP	US National Institute of Health Roadmap Epigenomics Project
NMDAR	n-methyl-daspartate receptor
NP	Neuropsychiatric
Nts	Nucleotides

OCD	Obsessive Compulsive Disorder
PANSS	Positive and Negative Syndrome Scale
PGC	Psychiatric Genomics Consortium
PKA	Protein Kinase A
PRC2	Polycomb repressive complex 2
RBBP4	Retinoblastoma binding protein 4 gene
RELN	Reelin gene
RNA	Ribonucleic Acid
rSNP	regulatory SNP
SA	South Africa/n
SASH	South African Stress and Health study
SD	Standard deviation
SAP18	Sin3A associated protein 18 gene
SGA	Second-Generation Antipsychotic
SIN3-HDAC	Sin3A histone deacetylase complex
SLC6A4	Serotonin transporter, solute carrier family 6 member 4 gene
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
SYNC	Syncoilin intermediate filament protein gene
SZ	Schizophrenia
TFBS	Transcription-factor binding site
UTR	Untranslated region
VKORC1	Vitamin K epoxide reductase complex 1
WES	Whole-exome sequencing
YLDs	Years-lived with disability

1. LITERATURE REVIEW

1.1. Introduction

Around 450 million people are burdened with a neuropsychiatric (NP) disorder, projecting mental disorders as one of the leading causes of detrimental health and disability globally (NMH Communications, 2001). Despite evident prevalence of these disorders worldwide, research is yet to unravel the complexities underpinning the causality, and subsequently adequate treatment development is hampered. Even though the considerable burden of these disorders, including adverse human, economic and social effects, has been made clear, efficient treatment of such disorders has failed to alleviate all such associated burdens (Kassebaum et al., 2016; Vigo et al., 2016). These disorders, including anxiety disorders and schizophrenia (SZ), emerged in the top 20 causes of global burden of disease in 2013 and was shown to reach epidemic proportions in low-income regions of the globe (Mayosi et al., 2009a; Vigo et al., 2016). Related behavioural, social, cognitive and perceptual disruptions are seen across individuals afflicted with SZ, Bipolar Disorder (BD), as well as both Obsessive-compulsive Disorder (OCD) and Autism Spectrum Disorder (ASD) (O'Connell et al., 2018a; Smeland et al., 2019). A vast overlap is seen in the clinical symptomology surrounding these disorders, for example; cognitive and perceptual deficits are shared between SZ, BD, ASD, and OCD whereby learning, mood, memory, perception and executive function are affected (Gilman et al., 2014; Krystal and State, 2014; O'Connell et al., 2018b).

Concurrent twin, family and adoption studies have identified that neuropsychiatric disorders like SZ and BD have a genetic basis and are influenced by environmental factors (Smeland et al., 2019). Much deliberation has been put forward regarding the proposed genetic model by which these disorders adhere and manifest, including the "heterogeneity model", the "common disease-common variant model" and more recently the "omnigenic model" (Yang et al., 2005; Peedicayil and Grayson, 2018). The "heterogeneity" model assumes that many individually rare genetic variants of large effect-sizes contribute towards the disorder, while the "common disease-common variant" model presents an opposing hypothesis that many relatively common variants of small effect-sizes interact in the manifestation of the disorder (Yang et al., 2005). The "omnigenic model" suggests that a vast number of causal variants with tiny effect-sizes occur widely across the genome and genetic contribution to disease is heavily clustered in regions known to be transcribed or marked by active chromatin (Boyle et al., 2017). With an increasing number of individuals reported as afflicted by at least one mental disorder, unraveling the genetic architecture of such disorders has taken precedence.

Shortcomings observed in treatment efficacy have also stimulated a shift in focus in genomics research regarding NP disorders (Drogemöller et al., 2014a).

1.2.Schizophrenia – incidence and impact

According to the most recent Global Epidemiology and Burden of Schizophrenia study, SZ accounts for 13.4 million years of life lived with a disability globally (Charlson et al., 2018). This multifaceted, heritable disorder affects approximately 1% of the global population. Despite this seemingly low incidence rate, SZ ranked as the 12th most disabling disorder amongst 310 diseases and injuries globally in 2016 (Charlson et al., 2018). In South Africa neuropsychiatric disorders like SZ rank third in contribution to overall disease burden ("Mental Health Policy Framework," n.d.).

1.3.Symptomology and progression

Schizophrenia is a chronic neurodevelopmental disorder, with varied symptomology divided into positive, negative and general/cognitive categories (Rund, 2018; Weïwer et al., 2013a). The positive symptomology most commonly associated with SZ are symptoms described as added to the normal behavioural repertoire, whereas the negative are a lack of or decrease in certain normal behavioural or developmental characteristics (Weïwer et al., 2013a). For instance, individuals afflicted with SZ may experience psychotic symptoms like hallucinations and delusions with impairments in speech, apathy and loss in motivation (Figure 1.1). Alongside these symptoms are considerable psychopathological symptoms like cognitive deficits and motor abnormalities. Collectively the symptoms and the severity of the disorder have an immense impact on the patients quality of life (Weïwer et al., 2013a).

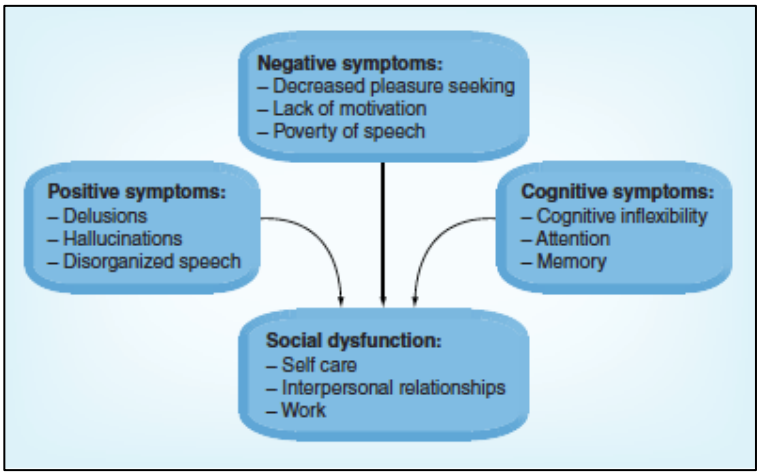

Figure 1.1 Symptomology of SZ and resultant impact on quality of life (Weïwer et al., 2013a). *Reprinted with permission from Newlands Press Ltd*

The Positive and Negative Syndrome Scale (PANSS) was developed to quantify these symptoms with regard to severity, response to treatment, relapse and remission of schizophrenia. Symptoms are measured categorically with severity determined numerically (Kay et al., 1987). This scale combined items of the Brief Psychiatric Rating Scale (Overall and Gorham, 1962) and the Psychopathology Rating Schedule (Singh and Kay, 1975) to assess both positive, negative and general psychopathology in complete definitions.

The onset of such symptoms is seen in adolescence to early adulthood however this onset and eventual progression is not always amenable to strict categorical classification (Parnas, 1999; Rund, 2018). These clinical features, age of onset and the course of illness including inter-episode recovery can vary widely amongst individual patients (Desbonnet et al., 2012). Despite difficulties in identifying predetermined phases of this disorder in its progression, efforts have been made to surmise epidemiological, clinical and phenomenological aspects of the evolution of SZ, into premorbid, prodromal, pre-psychotic to psychotic and psychotic to stable phases (Figure 1.2) (Parnas, 1999). The onset of this disorder is generally pinpointed by the first psychotic episode, followed by subsequent episodes separated by brief periods of remission (American Psychiatric Association and others, 2013). The so-called stable phase encapsulates lingering negative and cognitive symptoms with a general functional decline (Tandon et al., 2009).

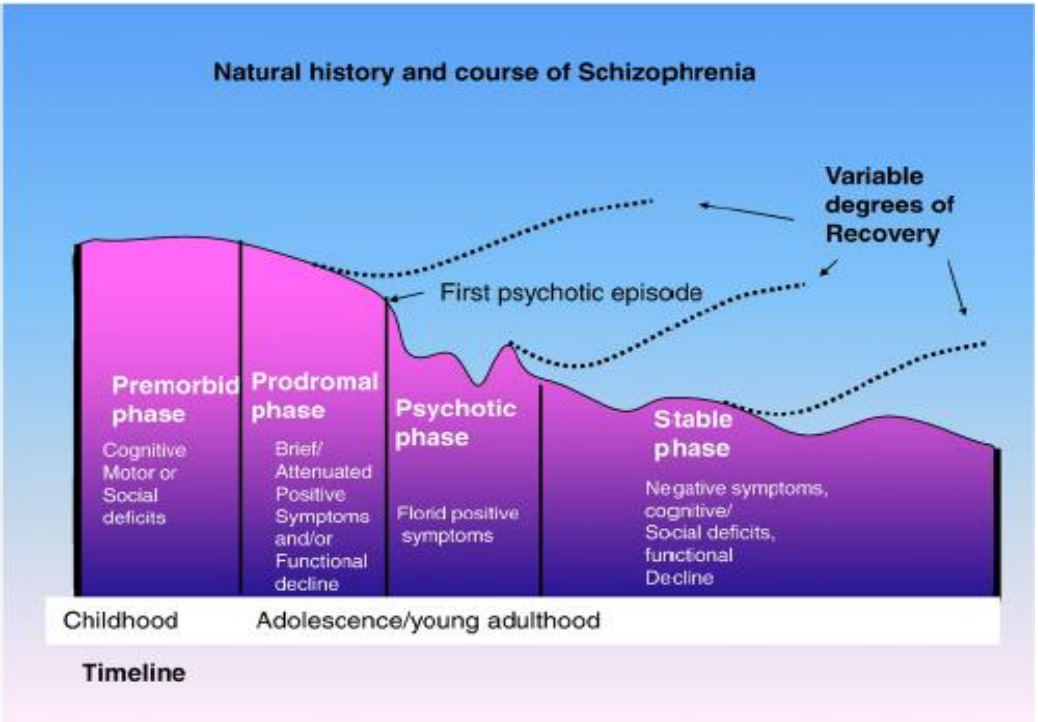

Figure 1.2 Progressive stages of schizophrenia (Tandon et al., 2009). *Reprinted with permission from Elsevier*

Although a standardised, absolute collective of symptomology at diagnosis is lacking, there is a general agreement regarding clinical characteristics in the expression of SZ (Figure 1.3). Given the associated symptomology and resulting functional impairments, SZ has been noted as one of the most debilitating mental disorders with regard to impact on both individuals and society (Tandon et al., 2009).

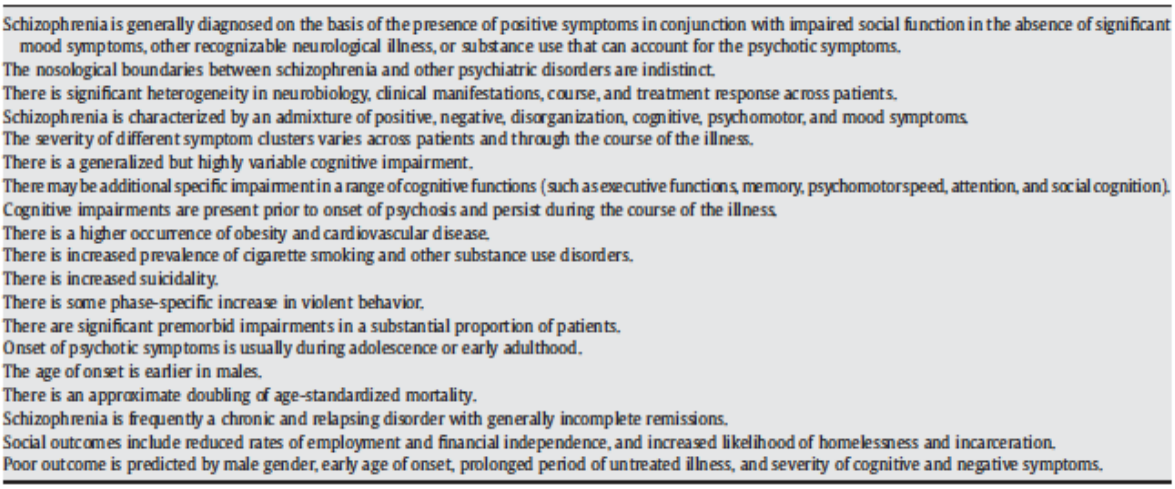
Schizophrenia is generally diagnosed on the basis of the presence of positive symptoms in conjunction with impaired social function in the absence of significant mood symptoms, other recognizable neurological illness, or substance use that can account for the psychotic symptoms.
The nosological boundaries between schizophrenia and other psychiatric disorders are indistinct.
There is significant heterogeneity in neurobiology, clinical manifestations, course, and treatment response across patients.
Schizophrenia is characterized by an admixture of positive, negative, disorganization, cognitive, psychomotor, and mood symptoms.
The severity of different symptom clusters varies across patients and through the course of the illness.
There is a generalized but highly variable cognitive impairment.
There may be additional specific impairment in a range of cognitive functions (such as executive functions, memory, psychomotor speed, attention, and social cognition).
Cognitive impairments are present prior to onset of psychosis and persist during the course of the illness.
There is a higher occurrence of obesity and cardiovascular disease.
There is increased prevalence of cigarette smoking and other substance use disorders.
There is increased suicidality.
There is some phase-specific increase in violent behavior.
There are significant premorbid impairments in a substantial proportion of patients.
Onset of psychotic symptoms is usually during adolescence or early adulthood.
The age of onset is earlier in males.
There is an approximate doubling of age-standardized mortality.
Schizophrenia is frequently a chronic and relapsing disorder with generally incomplete remissions.
Social outcomes include reduced rates of employment and financial independence, and increased likelihood of homelessness and incarceration.
Poor outcome is predicted by male gender, early age of onset, prolonged period of untreated illness, and severity of cognitive and negative symptoms.

Figure 1.3 Clinical characteristics of schizophrenia for diagnoses (Tandon et al., 2009). *Reprinted with permission from Elsevier*

In societal context, SZ is a very costly disorder; predominantly with regard to functional impairment and subsequent reduced productivity of sufferers, profound stigmatization as well as varied and inadequate efficacy of currently available treatment (Carr et al., 2004; Tandon et al., 2009). Although the course of SZ appears distinct, the exacerbations and remission periods are resolved with varying extent across individual sufferers during the course of illness (Andreasen et al., 2005; Haro et al., 2008) and those individuals suffering have a chronic struggle with both relapse and persistence of symptoms despite treatment provided.

1.4.Diagnosis

SZ is commonly diagnosed using the Diagnostic and Statistical Manual of Mental Disorders (DSM) in a clinical interview based on characteristics as outlined by the manual (American Psychiatric Association and others, 2013). This is not the only manual of its kind used in diagnoses, others include the International Classification of Diseases (ICD) (World Health Organization, 1992), with both manuals having high clinical diagnostic reliability. However, the most commonly employed is the DSM, throughout which the definition of SZ has evolved over six editions (DSM-I, DSM-II, DSM-III, DSM-III-R, DSM-IV, DSM-IV-TR and DSM-V) with the fourth edition notably having high clinical reliability and validity (Tandon et al., 2013, 2009). Despite the constant alterations to this

manual, three core concepts have contributed towards the definition of SZ; 1) the Kraepelinian accent on avolition, chronicity and poor outcome (Kraepelin, 1971), 2) the Bleulerian view on negative symptoms (Bleuler, 1950) and 3) the Schneiderian positive symptoms (Schneider, 1959). Given the reliability of the DSM-IV, diagnostic criteria have been carried over to the current version, DSM-V, however the heterogeneity of the disorder was poorly explained by the subtypes outlined in previous versions (paranoid, catatonic, disorganized, schizoaffective, undifferentiated, and residual) and has since been abandoned to preserve diagnostic reliability (Tandon, 2014). Characteristic symptoms of schizophrenia as retained from the DSM-IV are delusions, hallucinations, disorganized speech, exceptionally disorganized or catatonic behavior and negative symptoms like those described previously (section 1.3.). For a diagnosis to be made in the clinical interview, at least two of the five characteristic symptoms of SZ need to be met and present for a minimum 1-month period (Tandon et al., 2013).

The heterogeneity of SZ introduces another problem concerning diagnosis, as there is varied and significant clinical overlap between psychiatric disorders (O'Connell et al., 2018b). This is further complicated by excessive comorbidity observed amongst many mental disorders as well (Figure 1.4). Conflicting ideas and research have introduced discord in the narrative of defining mental disorders into distinct clinical categories, with many clinicians struggling to fit individual patients into neatly defined symptom 'boxes', suggesting rather a spectrum of 'dimensionality' to account for this overlap (Adam, 2013).

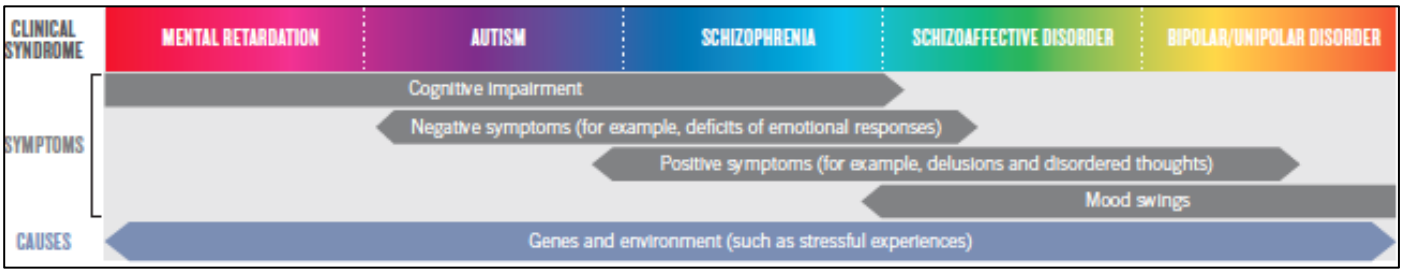

Figure 1.4 Proposed spectrum of mental illness in dimensional approach to diagnosis (Adam, 2013). *Reprinted with permission from Nature News*

The spectrum portrays mental illness as a dysregulation of normal processes and categorically separating these disorders may been seen as inhibitory towards research contributing towards the refinement of diagnoses processes. This phenomenon is described by comorbidities, *i.e.* the occurrence of two or more mental disorders in one individual, which is not all that uncommon with as many as 45% of patients manifesting mental illnesses in this manner (van Loo and Romeijn, 2015). Dichter and colleagues (Dichter et al., 2012) provided evidence of these overlapping DSM disorders

with anxiety and mood disorders resulting in part to hyperactivity of the amygdala region in the brain and those suffering SZ and post-traumatic stress disorder sharing aberrant activity in the prefrontal cortex. Given the insurmountable evidence regarding the prevalence of comorbidities, it seems fitting that the diagnostic tool would be in line with this representation and encapsulate all possible symptoms manifested across psychiatric disorders. This is a controversial and heavily debated view, however it cannot afford to be ignored given the current prevalence of mental illness and the lack of satisfactory answers to explain the high rates of comorbidity in psychiatric patients (van Loo and Romeijn, 2015).

1.5.Risk factors

1.5.1. Genetics

Given the complexity underlying disorders like SZ, it has been agreed that it is not the result of a simple single gene – unlike Mendelian disorders. This has given rise to hypotheses on the genetics associated with this polygenic disorder and disease manifestation in patients. Broadly speaking, polygenic disorders have been proposed to follow various models of transmission, as described previously (section 1.1).

The etiology of SZ involves various risk factors, broadly defined as genetic or environmental. Throughout the conceptualization of the disorder it became abundantly clear that the risk of developing SZ aggregated in family members, with an affected family member conveying considerably more risk as shown by twin and familial studies (Tandon et al., 2008b). These findings of course led to the interpretation of a genetic basis of disorders like SZ and hence heterogeneity explains the portion of variance liable for a disorder or illness accounted for by genetics (Tandon et al., 2008b). As epidemiological studies have progressed, SZ has gathered heritability estimates ranging from 60-80%, which are also influenced by environment (Hilker et al., 2018; Toulopoulou et al., 2019). Since the discovery of such links, multiple study designs and approaches have been developed and used in research. Clinical genetic studies identify the extent to which genetic underpinnings contribute towards disorder development. Chromosomal and linkage studies allow interrogation of placeholders in the genome where relevant risk genes may reside, association studies aim to identify variant modification to disorder risk and lastly knock-out studies allow for specific brain processes to be studied via this genetic modification (Tandon et al., 2008b), albeit this suggests the readiness of an animal model for the disorder.

The advent of molecular genetics saw linkage analysis studies emerge as the first DNA-based method aiming to discover genomic regions in affected extended or nuclear families and sibling pairs without

implication of specific allelic variants (Henriksen et al., 2017). These studies examine linkage in the form of co-segregation of genetic markers and predefined phenotypic traits whereby estimates of linkage between markers and the disease could be determined. This method is based on the physical linkage seen between sets of genomic loci on the same chromosome, which tend to be inherited together during meiosis (Henriksen et al., 2017). However, due to difficulties in replicability of linkage studies, it was determined that although SZ susceptibility loci were found harboring variants of importance, the loci themselves are not necessarily conferring risk (Badner and Gershon, 2002; Lewis et al., 2003; Ng et al., 2009). Hence this method proved impractical in power to address genomic loci with small effects and required cohorts of considerable size to address such issues (Risch and Merikangas, 1996).

The next molecular technique that attempted broadening the picture of SZ etiology was candidate gene approaches. These approaches utilized a case-control study design to identify potential, candidate susceptibility genes for the disorder and eliminated the problems experienced with linkage in detecting genes with small effect alleles (Henriksen et al., 2017). The majority of candidate genes have been selected due to their position or functionality with the mostly commonly cited amongst those being neuroregulin 1 gene (*NGR1*) (Mostaid et al., 2017), dysbindin gene (*DTNBP1*) (Yuan et al., 2016), the dopamine receptor genes (*DRD1 – DRD4*) (Talkowski et al., 2007; Hall et al., 2015), disrupted in schizophrenia 1 gene (*DISC1*) (Niwa et al., 2016) and the catechol-O-methyl-transferase gene (*COMT*), to name a few (Lewandowski, 2007; Matsuzaka et al., 2017). However, despite thousands of candidate genes being investigated in these types of studies the results as a whole have been underwhelming in translation to a better pathophysiological understanding of the disorder (Gejman et al., 2011; Haraldsson et al., 2011).

Perhaps the most suitable methodology for uncovering the genetic basis of SZ thus far has been that of association studies, like Genome-Wide Association Studies (GWAS) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), however these studies have only reported around 25% heritability for SZ (Anttila et al., 2018). In these hypothesis-free studies, variation in gene sequences spanning the genome can be compared between different phenotypic groups to identify loci associated with a specific trait. However, this approach comes with interpretive difficulties (Frelinger, 2015). The following genes and associated variants linked to etio-pathogenic relevance in SZ are important candidates in uncovering genetic contributors, *NGR1*, *DISC1*, *DTNBP1*, *DRD1 – DRD4* and *COMT*. Despite the identification of such associations, the etiology of SZ is known to be highly heterogenous and likely due to many genetic associations of small effect-sizes (Owen et al., 2005) and as a result impedes researchers abilities to perfectly replicate all

findings. Despite the advent of GWAS and its genetic contributions, most hits have not been identified in coding regions of the genome (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), indicative of an epigenetic component in the disorder pathophysiology.

The introduction of GWAS led study designs *a priori* of selected candidates, in a hypothesis-free manner allowing for interrogation of the whole genome in the hopes of identifying more informative genetic associations with disorder etiology. This unbiased approach uses the mapping of millions of single nucleotide polymorphisms (SNPs) facilitated by the International HapMap and Haplotype Reference Consortiums (1000 Genomes Projects), by which microarrays and chips facilitate scanning of said SNPs (International HapMap Consortium and others, 2003; Siva, 2008; Henriksen et al., 2017). The premise of GWAS is linkage disequilibrium, where non-random association of alleles occur at two or more loci and in this has been hypothesized that frequently occurring specific allele variants in patients may be indicative of genetic associations. Genome-wide significance has been established in these studies in attempts to diminish the occurrence of Type I errors or false positives, with a stringency set to $p < 5x10^{-8}$ (Henriksen et al., 2017). To date SZ-focused GWAS have largely failed to provide consistent support for the findings outlined by linkage and candidate gene studies but rather have provided insight to hundreds of susceptibility loci and traits with genome-wide significance and have been substantiated in meta-analytic replication studies (Ripke et al., 2013; Shi et al., 2009; The Schizophrenia Psychiatric Genome-Wide Association Study Consortium, 2011; Xiao and Li, 2016). Additionally, the Major Histocompatibility Complex (MHC) locus on chromosome 6 has been found repeatedly in such GWAS with SZ-genetic associations, which indicates this locus as the strongest in terms of significance. Famously the SZ working group of the Psychiatric Genomics Consortium (PGC) identified 128 independent SZ associations spanning 108 SZ risk loci, providing support for links between SZ, the dopaminergic system, and immune regulation (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). These GWAS findings also address issues that arose in other molecular genetic study approaches with the employment of massive consortia. Importantly GWAS has allowed for many novel SZ risk loci to be identified, each contributing in part to the observed heritability of the disorder.

The above findings in common alleles were thought to only attribute to about 1-2% of the genetic risk of the disorder (Zhang and Malhotra, 2013a). However, it has been estimated that only ~5% of genetic risk variance explained by common risk loci passing significance (Corvin and Sullivan, 2016). To date this common allele heritability is estimated to explain 25% of susceptibility for the disorder, however these risk loci fail to pass similar significance thresholds (Corvin and Sullivan,

2016). The shortcomings and limitations of GWAS are discussed further in context to this study below (section 1.11). The molecular genetic findings surrounding the polygenic nature of SZ have clearly indicated genetics as a strong risk factor for the disorder, however many of these approaches are accompanied by limitations and are based off of prior assumptions surrounding SZ transmission (Henriksen et al., 2017). Considering the clinical manifestation of SZ and its variability amongst patients, there is a clear indication of the need for incorporation of genetics into clinical conceptualization and diagnostics of SZ. As mentioned, the limitations of the current methodology leave much to be desired and the interrogation and interpretation of results need to be done so in a manner that eludes to the functional consequences and relevance to biological functioning of the individual (Sauer et al., 2007; McCarthy et al., 2014), which can be attained via a systems genetics approach in consideration of all contributing factors.

1.5.2. Environmental

As mentioned, environmental factors are known to interact with genetic variation in the etiology and manifestation of schizophrenia. These exposures include both biological and psychosocial risk factors from as early as antenatal and perinatal development periods through to childhood and adolescence and into early adulthood (Mäki et al., 2005). Links have been made between maternal infections and nutrient deficiency during the first and second trimester of pregnancy and an increased liability towards the development of SZ (Meyer et al., 2007; Penner and Brown, 2007). The exact neurobiological mechanism by which this risk is conferred is not clearly understood, however cytokines and aberrant immune responses to maternal infections have been seen to interfere with normal fetal brain development (Ashdown et al., 2006). Individuals having experienced childhood adversity are also at higher risk for development of the disorder (Schmitt et al., 2014). Socioeconomic stressors like urbanicity, migration, and socioeconomic status at birth were also found to increase risk towards SZ (Schmitt et al., 2014; Tandon et al., 2008b). Cannabis use during adolescence has also been linked to an increased risk for development of the disorder (Semple et al., 2005).

Arguably the most relevant inheritance model when considering gene-environmental interactions, for SZ is the “omnigenic model” in proposing a broader view of the genetic contributions underlying complex disorders like such. In the conceptualization of this model, Boyle *et al*., (2017) concluded findings to an extremely large number of common causal variants with small effect-sizes widespread across the genome, genetic contributions to disease markedly occur in regions transcribed by active chromatin and lastly that many of the ‘larger effect’ variants are modestly enriched in specific genes or pathways directly influencing disease. Despite these findings, it was consensus that the variants

contributing the most disease heritability tended to be spread across the genome and not in proximity to known disease-specific functioning genes, suggesting that perhaps the surrounding or "peripheral" genes to those considered as "core" genes in disease were important for disease heritability (Boyle et al., 2017). To substantiate their hypothesis, observations regarding contribution towards heritability of a disorder indicated that cell regulatory networks are highly interconnected and gives credence to any expressed genes regulating functioning of so-called core genes. These networks thus likely encapsulate all levels of interactions among cellular molecules, transcriptional networks, post-transcriptional modifications, protein-protein interactions and intercellular signaling (Peedicayil and Grayson, 2018). Newly developed molecular, technological and statistical methods over the last decade have sought to further uncover the genetic basis of SZ. Research efforts like the Human Genome Project brought much optimism in that determining the sequence of the entire genome would uncover previously unknown genetic variants associated with SZ (Henriksen et al., 2017).

1.6. Epigenetics

Epigenetic markers can be thought of as the molecular ties between external (environmental) and internal factors interacting to contribute to the clinical disorder manifestation. GWAS have made it abundantly clear that changes to DNA sequence are a crucial aspect of SZ disorder aetiology (Ovenden et al., 2018a), and the interplay between such alterations and the environment are mediated by epigenetic mechanisms. Epigenetics can be described as heritable change brought about in gene regulation and expression, exclusive of changes in DNA sequence (Bird, 2007). The umbrella term "epigenetic mechanisms" encapsulates those which regulate gene expression often leading to permanent changes that maintain stability throughout the lifetime of the organism and are potentially heritable (Goldberg et al., 2007; Portela and Esteller, 2010). These interactions result in differential clinical disorder phenotypes, however the mechanisms whereby these interactions take place are not fully understood. One such mechanism that has gained traction in literature though, is that of miRNA-mediated regulation.

Mechanisms as such are crucial in allowing for expression profiles which may adapt to a changing environment (Abdolmaleky, 2014). Regulatory mechanisms as such have been investigated in the genetic architecture of disorders and treatment response studies to a degree (Ovenden et al., 2017, 2018a). Signatures of epigenetic regulation resonate heavily throughout neurodevelopment, furthermore research on epigenetic dysfunction has shown evident impact in brain growth, synaptic plasticity, learning, memory and circadian rhythm (Borrelli et al., 2008; Mehler, 2008; Pidsley et al., 2010; Roth and Sweatt, 2009; Nakahata et al., 2007). This dysfunction has also understandably been

implicated in the development of psychiatric disorders like SZ (Ptak and Petronis, 2010). Growing evidence has shown links between regulatory variants, aberrant gene expression and NP disorders (Collins et al., 2010; Turner et al., 2016; Xiao et al., 2017; Yuen et al., 2016; Zhang and Lupski, 2015).

Maintaining transcriptional homeostasis is a fundamental regulatory mechanism for gene expression. Transcriptional homeostasis as described by Sallie (2004), is a proteins ability to modulate error incorporation (variability), with a subtle form of quality control seen exerted over protein synthesis (Sallie, 2004). This mechanism is fundamental to maintaining balance in cells. The regulation of RNA transcription and protein expression would be unsurprising in other roles like mediating immune escape and controlling cellular differentiation (Sallie, 2004). The following illustrates all factors in the system: (i) translated proteins interact with RNA polymerase; (ii) these interactions alter both polymerase processivity and fidelity; (iii) allowing wild-type protein or RNA polymerase interactions to be more avid and replicate alike RNAs more rapidly than mutant protein/RNA polymerase interactions (Sallie, 2004). Transcriptional dysregulation however, can be noted as any disruption/alteration or discontinuity in this process. In the context of this study, homeostasis can be viewed as the body's natural ability to respond to external and internal stimuli that work in disruption of a homeostatic alignment to biological and molecular functioning.

To date a sizeable amount of literature exists on the role of DNA methylation in psychiatric disorders (Teroganova et al., 2016). The clear and direct alterations seen by addition of methyl groups results in gene silencing with methylation identified in the psychopathology of many neuropsychiatric disorders like SZ as well as methylation target sites being identified for drug development (Denis et al., 2011; Grayson and Guidotti, 2013; Hendrich and Bird, 1998). Previous work has shown significant deficits in repressive DNA methylation affecting expression of the *GAD1* gene in individuals afflicted by SZ (Huang and Akbarian, 2007). Further investigations observed hypermethylation of post-mortem brain samples, at the promoter region of the *RELN* gene of SZ individuals (Abdolmaleky et al., 2005; Tochigi et al., 2008). Less literature on miRNA-mediated regulation in neurodevelopmental disorder manifestation can be found. These ~22 nucleotide (nt) long, abundant regulatory RNAs have been shown to have crucial roles in maintaining central nervous system functioning like neural differentiation and cognitive functions (Aksoy-Aksel et al., 2014; Sun and Shi, 2015; Woldemichael and Mansuy, 2016).

While epigenetic mechanisms have been identified to be involved in disorder pathogenesis the role of post-transcriptional mechanisms are not well understood (Du et al., 2019a). Such mechanisms like

those mediated by miRNAs have gained much traction in literature for their prevalent and global mechanism of action (Bartel, 2004; Du et al., 2019a; Mingardi et al., 2018). Widespread post-transcriptional effects on hundreds of genes by these endogenous RNA molecules exposes significant potential in disentangling the genetic architecture of etiology and treatment response in complex disorders like SZ. This too can further be substantiated by the following discussed insight to regulatory and noncoding regions of the genome (section 1.13), which may help address the missing heritability that is observed following GWAS and other approaches.

1.7. MicroRNA-mediated regulation

The identification of the first miRNA was in 1991 when an unusual deletion of two small sequences in the 3' untranslated region (3'-UTR) of the lin-14 messenger RNA (mRNA) in *Caenorhabditis elegans* was observed causing an accumulation of the protein (Wightman et al., 1991). No differences were observed in stability or functioning of the protein upon these deletions, suggesting post-transcriptional repression by binding of the respective mRNA (Wightman et al., 1991; Thomas et al., 2018). Since their initial discovery, multitudes of miRNAs have been identified, not only observed in worms, but in other animals and humans as well. These small, conserved regulatory mechanisms were observed to bind via complementary base pairing to target mRNAs and research focusing on them accelerated rapidly (Thomas et al., 2018). Proteins mediating miRNA synthesis were subsequently identified in parallel to elucidation of the RNA interface pathway, with the Dicer protein identified as the generator of mature miRNAs via cleavage of double-stranded miRNA precursors (Hutvagner, 2001). The Drosha enzyme was later identified to generate said miRNA precursors from longer primary transcripts and Argonaute proteins (Ago1-4) were seen to directly bind to miRNAs to mediate their effects on mRNA targets (Lee et al., 2003; Liu, 2004; Meister et al., 2004). The role of these small regulatory molecules soon became apparent, with critical implications in the development and function of the central nervous system (Thomas et al., 2018). Further identification of brain-enriched miRNAs in mouse brain (Lagos-Quintana et al., 2002) and expression in mammalian neurons provided the basis for suspected roles in neurodevelopment (Krichevsky, 2003; Miska et al., 2004). Early studies such as these provide evidence that miRNAs likely regulate brain development and neuronal functioning.

This regulation of brain development and neuronal functioning is brought about by the binding of respective miRNAs to the 3'-UTR region of their correlated target mRNA transcripts. Pinnacle studies identified the significant roles of these small RNAs in neural biology – illustrated by conditional Dicer ablation, miRNA depletion was observed in both neuronal progenitors and mature

neurons and subsequent impaired differentiation, function and neurodegeneration (Cuellar et al., 2008; Davis et al., 2008; De Pietri Tonelli et al., 2008; Kim et al., 2007; Schaefer et al., 2007). Positionally miRNA genes may lie within both intergenic spaces or introns/exons of protein coding genes (O'Carroll and Schaefer, 2013; Rodriguez, 2004). The binding of miRNAs to their targets does not necessitate perfect complementarity, nevertheless effects are mediated following site recognition as mRNA degradation or translational abnormalities (Thomas et al., 2018). The regulation of translation from mRNA to protein is mediated by miRNAs bound to a region that too has given insight to the effects of regulatory mechanisms. The binding of these small molecules to the 3' UTR and the mechanism that is actioned is known to contribute to synaptic plasticity in the mature brain, a process frequently disrupted in disorders like SZ (Aksoy-Aksel et al., 2014). The first evidence of this regulatory impact on plasticity was shown with miRNA-134 localizing to the postsynaptic compartment and locally regulating translation of *limk1* mRNA, which encodes a kinase with crucial implications for dendritic spine development in rats (Schratt et al., 2006). Importantly this study demonstrated how even a singular miRNA may have significant impacts on regulation and further, on neural circuitry.

Famously, miRNA-137 was identified to have roles in regulation of pre- and post-synaptic signalling, neuronal maturation and various forms of synaptic plasticity (Thomas et al., 2018; Sakamoto and Crowley, 2018). Moreover it was found that miRNA-137 dysregulation was associated with intellectual disability and SZ, implying a critical role in human brain functioning (Thomas et al., 2018). This miRNA is arguably the most well known in SZ research (Arakawa et al., 2019; Lett et al., 2013), with a GWAS revealing a novel SZ-associated SNP within the *MIR137HG* gene, along with another four loci known to harbour miRNA-137 binding sites (The Schizophrenia Psychiatric Genome-Wide Association Study Consortium, 2011). These findings were replicated by Kwon and associates (2013) and Ripke *et al*., (2013, 2014), with an additional SNP in *MIR137HG* (rs1702294) found to be second strongest in association to SZ by the latter research group (Kwon et al., 2013; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Notably both the identified SNPs minor alleles appeared to be protective against SZ (The Schizophrenia Psychiatric Genome-Wide Association Study Consortium, 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Further evidence was found in the DiGeorge 22q11.2 deletion, which is known to affect the key miRNA processing gene *DGCR8* and patients having said deletion showed a 30-fold increase in risk of SZ (Stark et al., 2008). In addition to these findings, SNPs occurring in other miRNA genes (miRNA-206, 198, 30e) were associated with SZ across different ethnic groups (Feng et al., 2009; Hansen et al., 2007; Xu et al., 2010). Not only have SNPs in miRNAs been associated with SZ, SNPs within miRNA binding sites (*i.e.* the 3'-

UTR) of SZ candidate genes were implicated in SZ risk when alteration to the binding site was resultant (Gong et al., 2013).

Case-control studies have led to the identification of differential expression profiles of circulating miRNAs in NP disorders like SZ (Du et al., 2019a; Geekiyanage et al., 2012; Gururajan et al., 2016; Maffioletti et al., 2016). In a SZ case/control study, 18 miRNAs were identified with significant expression changes between the groups and suggested potential diagnostically relevant biomarker miRNAs for distinguishing those with SZ from those without (Du et al., 2019b). The authors further suggested additional inquiry to the clinical relevance of such miRNA biomarker potential. The specificity of miRNA "hetero-silencing" determines regulatory impact across a vast number of genes (Bartel, 2004). In conjunction with the significant overlap observed in the genetic architecture of various NP disorders (both aetiology and ATR) it makes sense to further investigate the lengths to which this regulation influences the clinical manifestations that are seen (Calabrò et al., 2018; Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2019a, 2013; McGregor et al., 2019; Smeland et al., 2019). The SZ-working group of the PGC have explored the role of miRNAs in SZ risk genes in relation to the discovery of 108 genomic loci previously and provided evidence of miRNA in the aetiology of SZ (Hauberg et al., 2016a; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The role of miR-9-5p in particular was found to be implicated in functions of neurodevelopment and regulation of the dopamine D_2 receptor density. Additionally, 43 mature miRNAs were uncovered in SZ-GWAS loci (Hauberg et al., 2016b). Although inconsistent association of this group of miRNAs with SZ was found, the nature of their mechanism of action highlights potential influence in other areas of the disorder – such as ATR.

1.8.MiRNAs and pathways

Upon validation of targets like those of miRNA-137, bioinformatic analyses have revealed many SZ relevant pathways, like that of axonal guidance and ephrin receptor signalling, synaptic long-term potentiation (LTP) and protein kinase A (PKA) signalling (Wright et al., 2013). Although little to date is known about these specific pathways and others in SZ, the identification and evaluation of pathway-specific gene sets may allow for insight to consequential dysregulation of said pathways by miRNAs and how this confers risk (Wright et al., 2013). Alterations in activity patterns in neural circuitry supporting mental processing have shown a need for understanding the corresponding underlying biological pathways for complex conditions like SZ (Willsey et al., 2018). Considering miRNA-mediated regulation of gene expression, an aggregation of said regulated genes likely interact within biological pathways or at gene-network levels (Guo et al., 2010) and may offer an explanation

for missing heritability in NP disorders and associated traits. The findings supporting the "omnigenic model" of inheritance (section 1.1) too provides credence for miRNA-mediated regulation underlying complex disorders like SZ and associated traits. Transcription factors (TFs) and miRNAs may interact in one of two ways; either by reciprocally regulating one another forming feedback loops, or by both regulating their respective targets forming feed-forward-loops (FFLs). In an exploratory miRNA-TF mediated regulatory network analysis, FFLs as well as mutual feedback loops were identified in SZ (Guo et al., 2010). Well-documented candidate genes like *DISC1* are linked to SZ relevant pathways including the Wnt signalling pathway via inhibition of GSK3ß (Mao et al., 2009). Given how little is known about miRNA-mediated regulation in pathways relating to complex disorders and ATR, this is a potential avenue for interrogation of regulatory mechanisms, via their associated targets and subsequent pathway involvement. Therefore, potential insight to the consequence of dysregulation of miRNA-mediated regulation is also a possibility.

1.9.Treatment and response

The utilization of antipsychotic (AP) treatment for disorders like SZ, stands alone as the main method for management of the associated symptoms, where a single (monotherapy) or a combination (polypharmacy) of AP drugs can be used (Faries et al., 2005). One of the many challenges facing antipsychotic utilization is the limitation in alleviation of all pathological dimensions of the disorder for which treatment is necessary (*i.e.* negative, positive, and general/cognitive symptomology alleviation). Polypharmacy is usually as a last resort once all other monotherapy drugs have been exhausted in efforts to circumvent symptoms (Faries et al., 2005). Typically, the use of a singular antipsychotic is preferred when treating disorders like SZ, enabling the clinician to somewhat accurately monitor the patients response including avoidance of potential adverse side effects (Miller and Craig, 2002). The advent of AP drugs saw two classes of antipsychotics emerge; namely first-generation (FGA), and second-generation (SGA) or typical and atypical antipsychotics, respectively – whereby typical antipsychotics are often associated with extrapyramidal signs/symptoms (EPS) induced in an adverse response (Miller and Craig, 2002). Both classes of antipsychotics work to resolve positive symptoms associated with SZ, like delusions, hallucinations, and thought disorganization, however SGAs were considered by practitioners to be more effective with broader range of efficacy regarding negative and cognitive symptom domains as well (Tandon et al., 2008a). Non-response to medication is seen in ~20-30% of patients and only around half show favourable symptom improvement (Ackenheil and Weber, 2004; van Os and Kapur, 2009; Allen and Bishop, 2019). The comparative efficiency between these two classes of AP drugs is however highly debated,

though one fact remains despite the armamentarium of drugs available, the treatment of SZ and complete alleviation of symptoms is still unsatisfactory.

1.10. Adverse drug response

To date no AP agent has proved effective at alleviating symptomology across all domains, with inaccurate dosage decisions regarding prescription and henceforth treatment, are largely handled on a 'trial-and-error' basis (Tandon et al., 2008a). It is widely accepted however that SGAs have a lower inclination to induce EPS versus that of FGAs (Tandon and Jibson, 2002), however the SGAs have been associated with adverse metabolic effects (Tandon et al., 2008a). The risk of developing adverse drug reactions (ADRs) during treatment naturally introduces issues with patient compliancy. Metabolic side effects not only diminish the patients overall health outcomes, they can lead to serious risks like ischemic heart disease via antipsychotic-induced weight gain, dyslipidaemia and diabetes mellitus (Newcomer, 2005; Franciosi et al., 2005; Maciukiewicz et al., 2019). Accompanying these adverse metabolic effects are EPS, which are further subdivided into early-acute and late-onset EPS. Early-acute symptoms manifest at the beginning of treatment, or in alteration of treatment dosage and include akathisia (restlessness and pacing), acute dystonia (prolonged abnormal postures and muscle spasm) and parkinsonism (tremors, muscle rigidity and bradykinesia) (Mas et al., 2016). Given the nature of EPS, this class of adverse effects are heavily debilitating to the individual, stigmatizing and require additional pharmacotherapy to combat their occurrence (Divac et al., 2014). However very few studies into the proposed differences in FGA and SGA efficiencies have been done, of which the large-scale Clinical Antipsychotic Trials of Intervention Effects (CATIE) (Lieberman et al., 2005) and the Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia (CUtLASS) (Lewis et al., 2006b) studies found no significant observations supporting these claims. These studies did however provide evidence of clozapine having high levels of success in the treatment of refractory-SZ in particular (Lewis et al., 2006a). In combination with other measures, AP treatment has a significant impact on the course of illness (Figure 1.5) and thus optimal use, efficiency and avoidance of ADRs where possible is vital for SZ health outcomes (Tandon et al., 2008a).

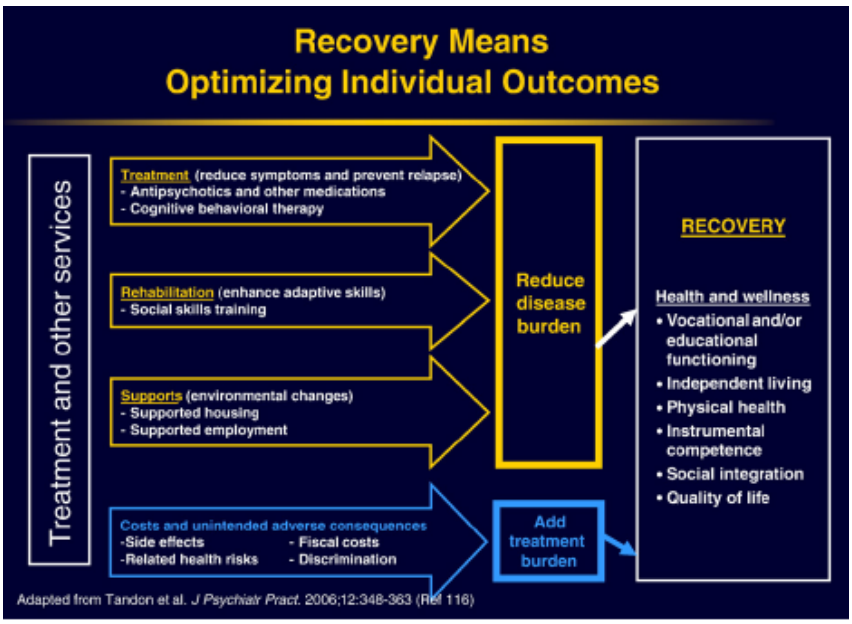

Figure 1.5 Antipsychotic treatment as key factor in schizophrenia recovery outcome (Tandon et al., 2008a). *Reprinted with permission from Elsevier*

1.11. Antipsychotic Treatment Response (ATR)

Interindividual variation is seen in drug response amongst patients indicating this as complex trait within SZ, in which the variation in genetic makeup relating to drug metabolism and neurotransmission, amongst other pathways, are factors (Blanc et al., 2010; Klein and Zanger, 2013; Ni et al., 2013). Investigation into patient's metabolic status evidence suggests a substantial role for genetic factors underlying this between-patient response variation. This was further substantiated by identified dopamine and serotonin receptor gene variants repeatedly associated with response phenotypes (Arranz and De Leon, 2007). Although methods for estimating patient response to antipsychotics are not standardized, the general approach is to use scales of symptom severity, like the Positive and Negative Syndrome Scale (PANSS) discussed previously (section 1.3) and to measure change in the respective symptom scale scores as indicative of treatment response for each symptom domain. Antipsychotic drug trials have largely utilized cutoff values in scales like the PANSS to define response, typically measured with baseline and post-treatment scores of these rating scales (Leucht et al., 2007). The definition of treatment outcomes has also been a challenge in accurately reporting ATR, with the ultimate goal of treatment aiming to maintain a state of remission devoid of relapse. The Remission in SZ-working group convened to develop criteria of remission states to accurately assess long term health outcomes for schizophrenia patients, enabling cross-study comparisons (Andreasen et al., 2005). These criteria state a low-mild symptom severity, where said symptoms do not impede a patients functionality and behavior (Frank et al., 1991).

Attempts at uncovering predictors of treatment response have long been a subject of research surrounding mental disorders like SZ, especially with the advent of AP treatment in the hopes of maintaining long-term outcomes like remission. Patients with poor insight towards their disorder underestimate or deny symptoms and the extent to which they are affected by the disorder and unsurprisingly are associated with reduced compliance and subsequent poor outcome. Importantly a factor like insight may be modified by pharmacological interventions and serves to predict potential relapse (Emsley et al., 2008). Another determinant of treatment response is duration of untreated psychosis (DUP), which displays potential for modification by pharmacological intervention. The shortening of the DUP period allows for improved treatment outcomes and can be seen inversely related to one another, where shorter DUP essentially equals a greater treatment outcome on the path to remission (Marshall et al., 2005; Perkins et al., 2005). As mentioned compliancy issues in treatment naturally impede treatment outcome, with non-adherence in patients resulting in a significantly higher risk for relapse (Robinson et al., 2004). However, issues surrounding non-adherence have been largely combatted with the introduction of long-acting injectables (LAIs), whereby other predictors, like early response have emerged. Asher-Svanum *et al*., (2011) evaluated early response to LAIs in attempt to find any association with improved overall response, qualifying as ≥40% improvement in symptomology (Ascher-Svanum et al., 2011). Early responders were defined as ≥30% improvement in PANSS scores by the fourth week and were predicted to experience significantly better clinical and functional outcomes for LAI overall response later. Lastly, the ability to identify patients failing to respond to initial treatment has proved to be a crucial predictor for patient health outcomes. The option of an alternative antipsychotic and earlier intervention can hence be considered in an attempt to prevent ensuing morbidity as a consequence of nonresponse (Chiliza et al., 2015a). It has been seen even in cases of standardized treatment that only a small percentage (23%) of patients responded to a secondary AP upon failure to respond to initial treatment. However more promising was the introduction of clozapine which saw a much larger group of patients (77%) with robust response, despite two failed treatment trials (Agid et al., 2007). It has therefore been proposed that an initial nonresponse can be predictive of subsequent nonresponse to another AP, other than clozapine (Remington et al., 2013).

Discrepancies in treatment response and interindividual variability can be attributed to the genetic constitution of an individual – although it has been shown that even this variability can only account for small interindividual differences, indicative of some non-genetic mechanisms acting on treatment response (Swathy et al., 2017; Ventola, 2013). High heritability and multifaceted genetic inheritance patterns of disorders like SZ, suggest that the interpretation of both common and rare variants from otherwise ‘unexplored’ genes (*i.e.* those not targeted in candidate gene studies) may reveal biological

mechanisms that are key in understanding disorder pathophysiology from ATR (Cross-Disorder Group of the Psychiatric Genomics Consortium and others, 2013). The identification of ATR as a complex trait then suggests that a considerable number of variants exist and ultimately interact to bring about these individual treatment response phenotypes that are observed (Ovenden et al., 2017).

Efficacy issues of antipsychotics naturally present compliancy issues for patients, which are further complicated by treatment strategy and variety, requiring optimization according to phase and severity of the disorder (Miyamoto et al., 2005). Primary focus on interindividual variation in drug efficacy has led to the development of pharmacogenetic strategies in the hopes of further developing pharmacogenetically-informed individualization of treatment. In addition, these strategies will aid in uncovering the potential underlying non-genetic mechanisms linking varied clinical manifestations and potentially explaining observed missing heritability in traits regarding disorders like SZ (Malhotra et al., 2012b). Considering the abundance of GWAS results available, investigation into noncoding regions of the genome is plausible, especially when noting how many results are reported occurring within these regions and whereby the associated SNPs are enriched for, or implicated in regulation (Maurano et al., 2012; Schaub et al., 2012). Pharmacogenomics has set out to uncover potential variants that may be interacting to bring about treatment response mechanisms, while considering a multi-directional relationship with common and rare genetic factors, environmental factors and gene-environment interactions like epigenetics (Manolio et al., 2009; Majchrzak-Celińska and Baer-Dubowska, 2017).

1.12. MiRNAs in ATR

Traction in literature over the last decade has led to investigation of miRNA involvement in NP disorders and various aspects of disorder pathophysiology like those discussed above, however investigation of their involvement in ATR has been limited. The regulatory effects of miRNAs are widespread, with the ability to regulate 20-30% of human genes (Lewis et al., 2005), highlighting potential roles in more than one avenue of disorder pathophysiology, with potential pathway disruption interacting in ATR mechanisms as discussed (section 1.11). Moreover the ability of miRNAs to target and therefore regulate, regulatory genes (i.e. *DNMT* genes in DNA methylation) introduces a potential regulatory cascade to be considered.

Importantly, Shi and colleagues illustrated both miRNA-9 and miRNA-326 mediated regulation of *DRD2* expression (Shi et al., 2014). The authors found that miRNA-326 overexpression reduced *DRD2* mRNA and *DRD2* receptor synthesis, however when observing antisense miRNA-326 and

miRNA-9, *DRD2* protein abundance was increased suggestive of endogenous repression by both miRNAs. Lastly, the variant rs1130354 residing within the *DRD2* 3'-UTR was seen to interfere with miRNA-326 mediated repression of *DRD2* expression. This study highlights the ability of miRNAs to regulate dopaminergic signalling. Considering that AP drugs directly interact with the dopaminergic system this provides substantial grounds to investigate miRNAs for potential influence in ATR. Another study shows an association between the down-regulation of miRNA-181b expression and improvement in negative symptoms of schizophrenia in a treatment naïve case/control cohort, constituting 20 SZ patients and 20 age-and-gender-matched controls. The expression of miRNA-181b, amongst others (miRNA-30e, miRNA-34a, and miRNA-7), was significantly higher in patients than in the normal control group (Song et al., 2014). A comparison was also made between pre- and post-treatment of the patient group, where miRNA-181b was found to be significantly downregulated, however four different antipsychotics were under examination in this study and so it cannot be determined which drug(s) were responsible for the observed changes in miRNA expression levels. Furthermore, studies examining expression of miRNAs in relation to ATR have not yet determined the potential roles that miRNAs may have in altering such responses, meaning characterization in a pathway-based, systems genetics approach may provide additional insights.

1.13. GWAS in pharmacogenomics

It was widely assumed that the utilization of GWAS upon its introduction a decade prior would unveil the genetic basis of complex disorders in their entirety, including implicated traits like ATR. It has become apparent via these study designs however, that casual factors may be more difficult to identify than originally thought and these shortcomings have given credence to different hypotheses of the genetic architecture of NP disorders like SZ and associated ATR as described above (section 1.11) (Gershon et al., 2011). Variants identified from disorder risk and drug target genes may have consequential impact as predictive markers on the individual level for predisposed risk and ATR (Kaur et al., 2014). Pharmacogenomics in this sense can be seen as a natural progression from the large-scale GWAS whereby the potential of variants to manipulate clinical symptoms and treatment response mechanisms has been identified (Kaur et al., 2014). The attempts at elucidation of genetic predictors of ATR has resulted in >500 pharmacogenetic publications examining the role of drug-metabolizing enzymes, neurotransmitter receptors, transporters and signalling molecules (Grossman et al., 2008; Gupta et al., 2013; Need et al., 2009; Paddock et al., 2007; Perlis et al., 2009, 2007; Zandi and Judy, 2010a), however, the exact mechanisms underlying the observed variance in ATR remain incompletely understood (Kaur et al., 2014).

This strategy of identification of genetic associations is achieved by genotyping markers, which when chosen effectively tag nearby common genetic variants, covering the entire genome with the premise of detecting associations between marker identified alleles and traits in samples of a given cohort (Gershon et al., 2011; Visscher et al., 2017). The detection of associations for GWAS to pharmacogenetics however proves difficult in terms of statistical power that is incurred when genotyping hundreds of thousands of small effect size SNPs (Cichon et al., 2009). This introduced the need of very large cohorts, to match the numerous amount of SNPs genotyped to effectively combine the considered effects of each SNP and lessen the burden of multiple testing (Zeng et al., 2015). GWAS chips have been developed with markers using the "common disease, common variant" basis as a working hypothesis, however these associations have failed to explain a substantial bulk of common disease inheritance, with limited clinical value (Gershon et al., 2011) and hence see research leaning towards alternate working hypotheses to meet these aims.

Currently GWAS has identified an abundance of genetic correlates and risk loci associated with commonly occurring NP disorders. Results from GWAS have been reported for an abundance of complex traits concerning major NP disorders like SZ, with results to date having likely revealed >200 SZ risk loci (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Ripke et al., 2011, 2013; Yu et al., 2018; Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2019). Despite these promising results and polygenic inheritance that too can account for some portion of this missing heritability (International Schizophrenia Consortium, 2009), further interrogation of these results are required to uncover attribution to the total missing heritability observed. While the main focus of GWAS has been uncovering disorder etiology and causal factors, an important contribution to the management of NP disorders. Treatment response has often been overlooked. Multifactorial traits interact with treatment efficacy and pharmacogenomics has moved in the direction of unravelling genetic architecture from disorder aetiology, associated traits like treatment response. These approaches now aim to contribute towards the observed 'missing heritability' component that may be underlying aspects of ATR in complex disorders (Ovenden et al., 2018b).

Given the limited clinical utility of most GWAS results, pharmacogenetic/genomic approaches were designed to disentangle disorder etiology from ATR, with the hopes of identifying reliable and clinically meaningful predictors of treatment response (Malhotra et al., 2012c). The terms pharmacogenetic and pharmacogenomic are often used interchangeably, although some have denoted the -genetic aspect to referring to singular genes under study versus the whole genome implied by -genomic (Goldstein et al., 2003). In its entirety it is the relation of heritable variation to interindividual

variation in drug response. Pharmacogenomic studies have great appeal when considering heterogeneity and prognostic uncertainty seen in most NP disorders, offering steps towards individually tailored treatment for ATR. However, many pharmacogenetic studies are using a candidate gene approach, the majority of which are centred around receptor pharmacology of the psychotropic agent (Arranz and De Leon, 2007; Fleeman et al., 2011; Malhotra et al., 2012c).

The roles of these genes can be further divided into pharmacokinetic and pharmacodynamic, where the former refers to the processes involved in drug metabolism and the latter to interactions between the drug, transporters and target molecule(s) (Zandi and Judy, 2010b). Pharmacokinetic studies are supported by identification of multiple functional variants with well characterized effects on drug metabolism, for example the >100 SNPs in the gene for the cytochrome P450 2D6 enzyme that have been associated with numerous drugs and metabolizer phenotypes (Ingelman-Sundberg, 2005; Ito et al., 2018). Many of these variants produce non-functional or reduced-function enzymes and led to the classification of four *CYP2D6* phenotypes of enzymatic activity in drug metabolism; namely poor, intermediate, extensive and ultra-rapid metabolizers (Malhotra et al., 2012c). Although these classifications have been made, there is little evidence to merit the relationship between pharmacokinetic variation and drug dosage, however in prevention of ADRs some may advise on dosing according to *CYP450* genotype (de Leon et al., 2006). Upon discovery of clozapine's efficacy in treatment, many pharmacodynamic studies have investigated the interaction between this agent and the dopaminergic and serotonergic receptor systems (Arranz et al., 2011). The targeting of these systems has proved crucial for mediation of antipsychotic effect, with an association between the dopamine D_4 receptor mediating efficacy of clozapine in SZ-patients otherwise deemed refractory (Zhao et al., 2005), alongside association of variation in the 5-HT_{2A} receptor with clozapine treatment outcome (Arranz et al., 1995; Yu et al., 2001). These studies have provided much insight to the plethora of potential mechanisms of antipsychotics in treatment response, yet there has not been a distinct improvement in overall ATR thus far.

Given the best efforts of multiple GWAS for many complex disorders, even outputs that are seemingly notable (*i.e.* identification of 108 independent genomic SZ risk loci with genome-wide significance) fall short in explanation of the causality, etiology and treatment outcomes (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Interestingly some SZ risk loci identified in GWAS were found to be over-represented in active regulatory regions in the brain and also appeared enriched in genes regarding postsynaptic density, postsynaptic membrane, dendritic spine, axon and voltage-gated calcium channel pathways (Gusev et al., 2014; Roussos et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Alongside

the lack of improvement in ATR despite pharmacogenomic studies, alternative systems genetics, hypothesis-free approaches may lead to the answers that were not identified using these prior approaches. GWAS have divulged a wide spectrum of common variation, both in coding and non-coding regions, with the latter found associated with disorder phenotype and consequential regulatory impact (Liou et al., 2012; Clark et al., 2013). The underlying pathophysiology of NP disorders like SZ may also be resultant of an interplay between both genetic and environmental factors. Therefore predictors of disorder etiology can potentially be uncovered when investigating individual genotypes in combination with respective environmental stressors (Roth et al., 2009).

With the above knowledge gap identified and the clear potential for regulatory influence in SZ, I hence decided to investigate further this role that miRNAs may facilitate in the manifestation of differing ATR outcomes. The lack of research surrounding associated traits like ATR and heavy socioeconomic burden that South Africa currently faces further motivated this study.

1.14. Current study

1.15. Aim and objectives

This study aims to characterize the role of miRNA-mediated regulation of genes in the aetiology of antipsychotic treatment response, in a South African FES cohort.

The specific objectives by which the aim will be achieved are as follows:

1. Candidate gene approach

Characterization of variation (novel and previously described) within candidate regulatory genes from the Taqman Human DNA methylation and transcriptional repression microarray (Applied Biosystems, California, USA).

a. Identify variants by literature search, online databases for population-specific variation, eQTL databases and available whole exome sequencing for 11 MA FES patients.
b. Identify SNPs which create or abolish miRNA regulatory sites using *in silico* tools.
c. Prioritize variants for investigation using *in silico* bioinformatic tools.

2. miRNA-target approach

Characterize miRNA binding sites/target genes utilizing *in silico* analyses, for 43 mature miRNAs as identified by Hauberg *et al*., (2016).

a. Identify gene targets for the identified miRNAs using gene ontology and biological pathway-based approaches.
b. Identify variants annotated by known regulatory elements (TFBS and miRNA target sites)
c. Correlate *in silico* predicted miRNA effects with online and available expression databases.

3. Impact of identified SNPs and gene targets in the context of miRNA-mediated regulation and antipsychotic treatment response

Investigate whether the prioritized miRNA-mediated SNPs/genes are associated with antipsychotic treatment outcomes in the SA FES cohort.

a. Perform linear and/or logistic and mixed-effect regression analyses to identify associations of variants with antipsychotic treatment outcome as defined by a change in PANSS scores over time.
b. Perform pathway analyses to identify potential miRNA-mediated regulatory impact in a systems genetics approach.

2. METHODS AND MATERIALS

Overview of the strategy described by the objectives of this study can be seen below.

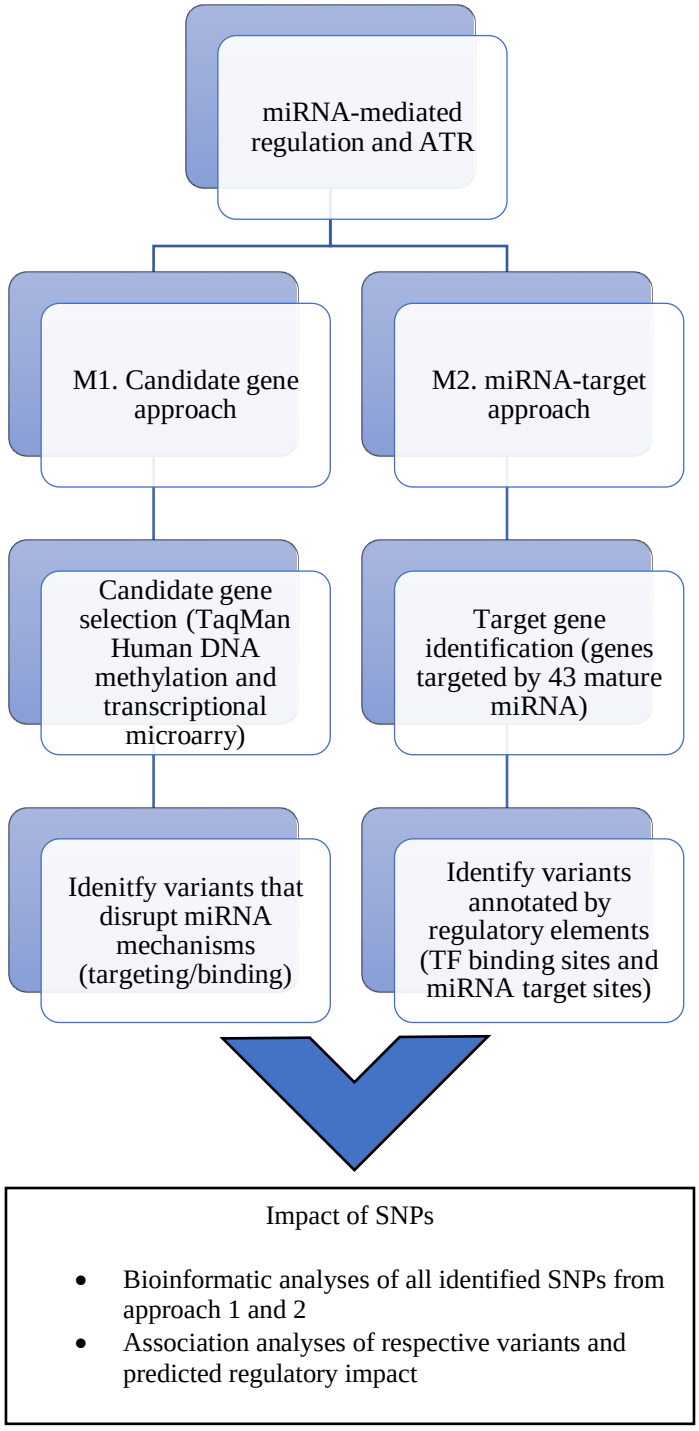

Figure 2.1 Proposed strategy outline of current study; M1: method 1; M2: method 2.

2.1. Cohort demographics

A previously recruited and described cohort of unrelated South African (SA) first episode schizophrenia (FES) patients was utilized (Drogemöller et al., 2014a; Chiliza et al., 2015b). The

cohort comprised of 103 previously drug-naïve patients, including 82 individuals of South African Mixed-Ancestry, 13 Xhosa, and eight Caucasian individuals. These individuals were represented by 27 females and 76 males with a mean age of 24 years (standard deviation (SD): ± 6.71 years). Patients were recruited from Stikland Hospital. Both written and verbal consent was obtained from the patients or their respective caregivers where applicable. Clinical assessments were performed at nine different time intervals; fortnightly for the duration of 6 weeks, and then every three months thereafter (Drogemöller et al., 2014b). During assessments ongoing patients received an injectable first-generation antipsychotic (FGA), *i.e.* Flupenthixol Decanoate (Fluanxol®, Lundbeck, Copenhagen, Denmark), bi-weekly over a period of 12 months. All clinical data, inclusive of PANSS scores, was obtained as well as the appropriate demographic information (age, gender, ethnicity). Additionally, the 103 patients were further stratified based on treatment-response outcome groups represented by nine individuals experiencing treatment refractoriness, 16 having relapsed, and 66 said to have experienced extrapyramidal adverse events (EPSAE) during the duration of treatment.

2.2. Inclusion and exclusion criteria

At the time of recruitment individuals were diagnosed using the fourth edition of the Diagnostic and Statistical Manual of Mental Diseases (DSM-IV) (APA, 1994) for schizophreniform disorder, schizoaffective disorder, or schizophrenia, having not been exposed to antipsychotic treatment for a maximum of a 4 week period during their lifetimes (Chiliza et al., 2015b). Inclusion criteria was based on the patient's completion of three months of treatment with no relapse occurrence. Relapse were defined as a 25% increase in PANSS total scores, an increase of 10 points when PANSS total is less than 40, or clinical deterioration (CGI change score of six or seven). Individuals were excluded based on current substance abuse usage, previous treatment by long-acting injectable antipsychotic(s), and significant physical illness or mental retardation (Chiliza et al., 2015b).

2.3.Genotype data

Blood samples were taken and DNA extracted for genotyping. Genotyped data was made available for the entire cohort, attained using the Illumina HumanOmniExpressExome BeadChip (Illumina, California, USA), for ~1 million SNPs. Resultant genotyped SNPs were used for analyses. The inclusion of ancestry informative markers (AIMs) for correction of population stratification was made due to the nature of the patient ethnicity groupings (Daya et al., 2013). Hence correction for population stratification is necessary. Genotyping for 100 AIMs previously designed for the South African Mixed-Ancestry population were included for all FES patients (Daya et al., 2013).

2.4.Ethical consideration

Ethical approval was obtained from the Health Research Ethics Committee, Faculty of Medicine and Health Sciences, Stellenbosch University (N06/08/148).

2.5.MiRNA mediated regulation and ATR

2.5.1. (M1) Candidate gene approach

In the hopes of providing greater insight of the underlying mechanisms influencing ATR in SZ, miRNA-mediated gene regulation was investigated in this study. A novel bioinformatic pipeline was utilized to identify genetic variants with known predicted effects pertaining to miRNA target and binding sites. This study design included a candidate gene approach (M1) and a hypothesis-free miRNA-target approach (M2). The candidate gene approach was based on previous work identifying the potential role of regulatory genes in ATR using a methylation and transcriptional repression microarray (O'Connell et al., 2019). However, considering all genes are subject to regulation by regulatory mechanisms, regulatory genes are no exception with regulatory influence from mechanisms like those mediated by miRNAs. The results of M1 will be further investigated via statistical association analyses and bioinformatic pathway analyses. The M2 results will be investigated via enrichment analyses and compared to the aforementioned as validation. The bioinformatic approach to be run in parallel for both approaches are outlined in Figure 2.2 below. All bioinformatic tools were used independently and no prior data was utilized in this study.

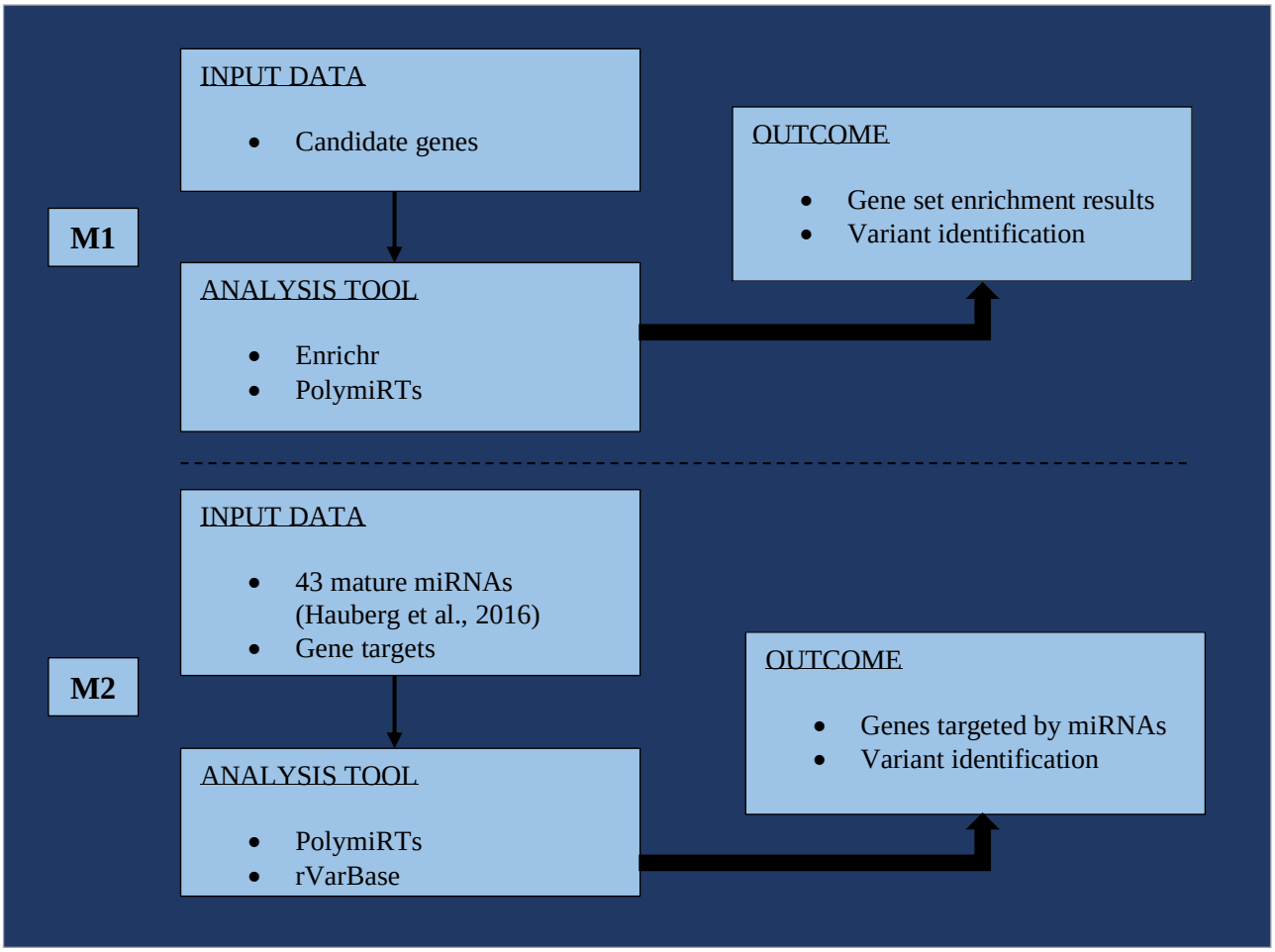

Figure 2.2 Summary of bioinformatic pipeline flow used in this chapter, the results generated in the outcome will be further processed by Association Analyses and pathway interaction investigation respectively.

2.5.2 Candidate gene selection

Candidate genes were chosen from those described on the microarray aforementioned (appendix, Supplementary Table 1), in subsequent prioritization steps. The gene set enrichment analyses tool Enrichr (Chen et al., 2013; Kuleshov et al., 2016), was employed to assess the relevance of these genes in terms of predicted miRNA gene targets, as well as biological and molecular processes/functions relating to regulatory mechanisms, as well as any previously reported associations to SZ and related implicated neurological processes or previously described neurological impairments. Briefly, eight databases were chosen in relation to genes identified as miRNA gene targets, those with pre-identified implications in neurological processes and those relating to translation or transcription in any way. The candidate genes that were selected had to occur across ~90% of the selected databases.

2.5.3 Variant prioritization

Variants were identified within the selected candidate genes using PolymiRTs (Bhattacharya et al., 2014) and were prioritized based on a prediction to interfere with miRNA-binding sites. All variants within these genes were considered for further analyses. A literature search was also performed to identify any known or commonly occurring variants which were included for comparative analysis with those already identified by PolymiRTs. The following key words/phrases were used in a manual search (April – May 2018) across PubMed (https://www.ncbi.nlm.nih.gov/pubmed/advanced) and Google Scholar (https://scholar.google.co.za) respectively; *schizophrenia*, *pharmacogenetics*, *antipsychotic treatment response*, *miRNA-mediated regulation*, *signaling pathways*.

All relevant variants in the First-Episode Schizophrenic (FES) cohort's SNP data were extracted using PLINK v1.90 (http://pngu.mgh.harvard.edu/purcell/plink). The FES data is described below.

2.5.4. Bioinformatic analyses

Predicted effects annotated by PolymiRTs were determined for the identified FES SNPs. Predicted functional and regulatory impacts of the variants chosen for further statistical and association analyses were performed using the online tool RegulomeDB™ for known and predicted regulatory elements in the intergenic regions of the human genome (Boyle et al., 2012). The predicted regulatory DNA elements include regions of DNAse hypersensitivity, transcription factor binding sites (TFBS), and promotor regions that have been biochemically characterized to regulate transcription. This tool sources data amongst public datasets from GEO (Barrett et al., 2012), the ENCODE project (Davis et al., 2018) and published literature. Next the tool rSNPBase (Guo et al., 2014a) was used to annotate the SNPs regulatory capacity with reference to experimentally supported regulatory elements (Guo et al., 2014a). rSNPBase focuses on regulatory SNPs (rSNPs) involved in various regulation types, including proximal and distal transcriptional regulation and post-transcriptional regulation, within the input genes. Spatio-temporal labels and experimental eQTL labels for SNPs are also provided. Allelic frequencies were investigated comparatively to Ensembl minor allele frequencies of the African, European, South Asian and East Asian populations. Genotypes and frequencies for the respective variants were also determined in the FES cohort, and compared to the frequencies within 1000 Genomes populations (Clarke et al., 2017).

2.5.5. Association analyses with ATR

The identified variants that presented with a MAF ≥0.05 were used in the association analyses. Association analyses were performed in R (R Core Team, 2018) using the R packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017). Linear mixed-effects models were used for the investigation of the effect of genetic variants on change in PANSS scores for each respective subscale (positive, negative and general) and total over the 12-month period. Adjustments were made for age, gender, proportion ancestry (using AIMs markers) and baseline PANSS scores. This study is only concerning cases and hence associations calculated for respective variants and treatment outcomes. Indicators of treatment response are given as percent changes, observed for the respective symptom domain (ΔPANSS over time). An observed positive change (percentage change values more than zero) is indicative of worsened ATR and negative (percentage change values less than zero) changes indicative of an improved treatment outcome over time.

Both a genotypic and an additive mode of inheritance were investigated, with the inclusion of a genotypic model to account for potential heterozygous advantage, whereby selective preference for a heterozygous phenotype of higher fitness can occur (Charlesworth and Willis, 2009; Penn et al., 2002). Bonferroni correction was used to correct for multiple testing. This resulted in a threshold value of $p \leq 1.04 \times 10^{-3}$ (0.05 / (4 tests x 6 SNPs).

2.5.6. Bioinformatic pathway analysis

The identification of variants significantly associated with antipsychotic treatment outcomes, allowed for investigation of pathway and network interactions. These interactions may highlight underlying miRNA-mediated regulatory mechanisms interacting in a system potentially responsible for the manifestation of interindividual treatment response. The bioinformatic tool NetworkAnalyst (Xia et al., 2014, 2013b, 2013a; Zhou et al., 2019) was used for visual analytics for gene expression profiling and interrogation of network processes of interest. NetworkAnalyst (available at: https://www.networkanalyst.ca) was used to interrogate pathways in relation to gene-miRNA interactions and transcription factor-miRNA coregulatory network interactions. The NetworkAnalyst tool can be used to perform meta-analyses of gene lists or gene expression datasets labeled with multiple metadata parameters, or finally of multiple independent gene expression datasets. The data integration is achieved via robust statistical procedures and visual output to be explored within protein-protein interaction (PPI) networks, interactive heatmaps or chord diagrams (Xia et al., 2015).

The four relevant ATR associated genes; *SAP18*, *HDAC2*, *HDAC4*, and *HDAC5* were used as the gene list input for the meta-analysis and network visualization. During the analyses, summary-level data (P-values, fold changes (FCs) or effect sizes) is made available for user download as well, it is extracted and integrated to identify genes that are significantly altered in expression based on the overall evidence. The given input genes/drugs/chemicals are denoted as nodes, with edges classifying interactions between given nodes. Subnetworks are created as sets of connected nodes and edges. The p-value of a module is determined solely on network connectivity and gives an indication of how significant the connections within a defined module are. The edges within a module are denoted internal and edges connecting nodes of a module with the rest of the graph denoted external. Generally, the null hypothesis of the test states there is no difference between the number or internal and external connections to a given node in the module. The p-value is therefore calculated using a Wilcoxon rank-sum test (Wilcoxon, 1945) of the internal and external degrees. Selected genes here are presented within PPI networks. In the absence of gene expression data only a singular analysis can be performed as outlined in the workflow below (Figure 2.3). For the purpose of this study and given the nature of the predetermined microarray from which these genes were initially selected from, only miRNA-relevant analyses were applicable.

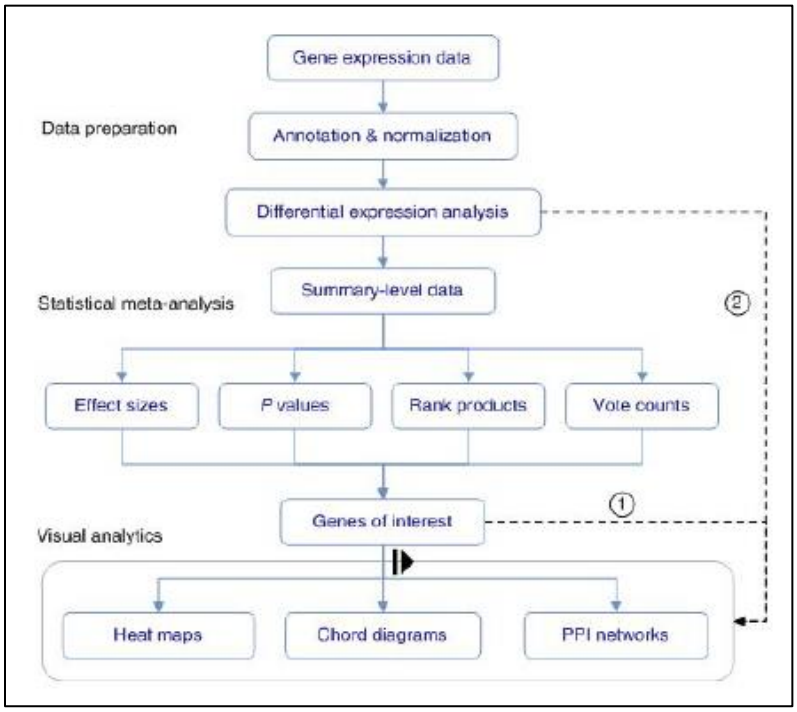

Figure 2.3 Design workflow of NetworkAnalyst (Xia et al., 2015). *Reprinted with permission from Springer Nature*

2.6. (M2) MiRNA-target approach

2.6.1. MiRNA gene target identification

The gene targets for 43 mature miRNAs (appendix Table 2) were identified using the online predictive tool PolymiRTS (Bhattacharya et al., 2014), the database of DNA variation occurring in miRNA seed regions and target sites, with predicted implications for miRNA-miRNA interaction and hence miRNA-mediated gene expression (Bhattacharya et al., 2014). The database can predict targeted genes of miRNAs of interest.

2.6.2. miRNA target gene variant prioritization

The bioinformatic tool, rVarBase (http://rv.psych.ac.cn), a database for regulatory features of human variants with regulatory features in three fields: chromatin state of the region surrounding variant, regulatory elements overlapped with variant, and variant's potential target genes (Guo et al., 2016), was used for the target gene variants to be prioritized. A literature search was also performed to ensure that any known or commonly occurring variants were included for comparative analysis to those identified by rVarBase. The same key words/phrases as described above, were used in searches across PubMed (https://www.ncbi.nlm.nih.gov/pubmed/advanced) and Google Scholar (https://scholar.google.co.za), during April – May 2018. Variants were extracted from the whole-genome data of the FES cohort (described later) using PLINK v1.90 (http://pngu.mgh.harvard.edu/purcell/plink).

2.6.3. Bioinformatic pathway analysis

After the identification of genes targeted by the previously described mature 43 miRNAs (appendix, Supplementary Table 2) the genes were analyzed by Enrichr to categorize and identify relevant pathways according to gene ontology analysis as provided by this tool. Enrichr provides context of 35 gene set libraries partitioned across six categories (pathways, ontologies, transcription, diseases/drugs, cell types and miscellaneous) for a library of genes. Enrichment is calculated using Fishers exact test (Fisher, 1970) assuming a binomial distribution and independence for probability of any gene belonging to any given set. The Z-statistic is utilized in determining the corresponding corrected p-values (Chen et al., 2013). The resultant pathways were stratified in the results by any relation to regulatory mechanisms, gene expression, pathways known with prior implication in SZ and/or reported relation to disease.

The results of the gene set enrichment analyses were separated into lists pertaining to genes shared across categorical databases and genes which were seen to be 'unique' (*i.e.* only appearing once) across databases. The top 20 gene enrichment categorical results displayed for the respective target genes are available in the appendix (Supplementary Tables 3 – 15). The bioinformatic pathway analyses result from M1 above was investigated in comparison to the enrichment output to confirm results and identify potential overlaps.

Furthermore, an abundance of variants, including ~535 FES SNPs, were identified within the target genes of the 43 miRNAs. The Functional Mapping and Annotation of Genome-wide association studies (FUMA) tool (https://fuma.ctglab.nl) was used to annotate and interpret the SNPs in a SNP2GENE and GENE2FUNC analysis. SNP2GENE works to annotate either GWAS summary statistics or SNPs as input, functionally, followed by GENE2FUNC with the resultant input for biological context. The FUMA pipeline is described by the figure below, with the respective process used in this study outlined (Figure 2.4) (Watanabe et al., 2017).

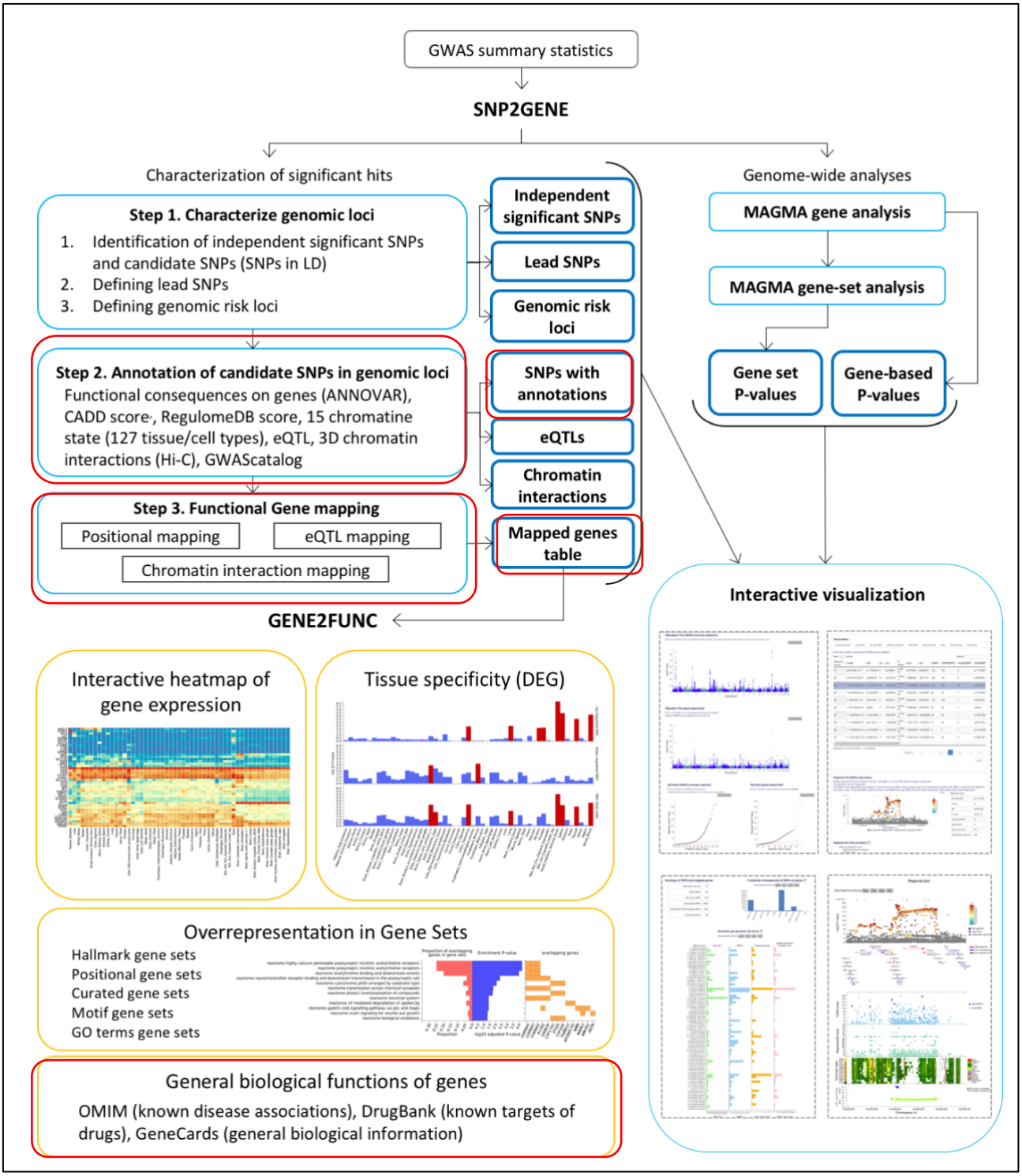

Figure 2.4 FUMA pipeline (https://fuma.ctglab.nl/tutorial), with highlighted processes used outlined in red.

3. RESULTS

3.1. (M1) Candidate gene selection

The microarray gene selection was prioritized by gene set enrichment, the relevant results of the appropriately selected databases and the corresponding categorical enrichment gene sets from Enrichr are shown below (Table 3.1). Those pathways with pre-identified implications in neurological processes and those relating to translation or transcription in any way were selected as seen below.

Table 3.1 Predicted gene enrichment categories for candidate gene selection as defined by Enrichr

Gene Set Enrichment Analysis			
Database	**Category/Target/Pathway**	**p-value**	**Genes**
GO Molecular Function	Histone deacetylase activity H3-K14 specific	7.495e-29	*MBD3*, ***HDAC4***, ***HDAC5***, ***HDAC2***, ***HDAC3***, *HDAC10*, ***HDAC1***, *HDAC11*, *CHD4*, ***HDAC8***, ***HDAC9***, ***HDAC6***, ***SAP30***, ***RBBP4***, ***SIN3A***, ***SAP18***, *RBBP7*
Reactome	Epigenetic regulation of gene expression	7.114e-21	*MBD3*, *DNMT1*, ***HDAC2***, ***HDAC1***, *DNMT3A*, *MBD2*, *CHD4*, *ACTB*, ***SAP30***, ***RBBP4***, ***SIN3A***, *DNMT3B*, ***SAP18***, *RBBP7*
Wikipathways	Neural crest differentiation	5.710e-16	***HDAC4***, ***HDAC5***, ***HDAC2***, ***HDAC3***, *HDAC10*, ***HDAC1***, *HDAC11*, ***HDAC8***, ***HDAC9***, ***HDAC6***, *HDAC7*
GO Biological Process	Negative regulation of transcription from RNA polymerase II promotor	4.777e-10	***HDAC4***, *MBD3*, ***HDAC5***, ***HDAC2***, ***HDAC3***, *HDAC10*, ***HDAC1***, *MBD2*, *CHD4*, ***HDAC8***, ***HDAC9***, ***HDAC6***, *MECP2*, ***SIN3A***, *DNMT3B*, ***SAP18***
GO Molecular Function	Histone deacetylase activity	2.842e-25	*MBD3*, ***HDAC4***, ***HDAC5***, ***HDAC2***, ***HDAC3***, *HDAC10*, ***HDAC1***, *HDAC11*, *CHD4*, ***HDAC8***, ***HDAC9***, ***HDAC6***, ***SAP30***, ***RBBP4***, ***SIN3A***, ***SAP18***, *RBBP7*
	Histone deacetylase activity H4-K16 specific	2.434e-28	*MBD3*, ***HDAC4***, ***HDAC5***, ***HDAC2,*** ***HDAC3***, *HDAC10*, ***HDAC1***, *HDAC11*, *CHD4*, ***HDAC8***, ***HDAC9***, ***HDAC6***, ***SAP30***, ***RBBP4***, ***SIN3A***, ***SAP18***, *RBBP7*
	NAD-dependent histone deacetylase activity	2.248e-28	*MBD3*, ***HDAC4***, ***HDAC5***, ***HDAC2***, ***HDAC3***, *HDAC10*, *HDAC1*, *HDAC11*, *CHD4*, ***HDAC8***, ***HDAC9***, ***HDAC6***, ***SAP30***, ***RBBP4***, ***SIN3A***, ***SAP18***, *RBBP7*
	Histone deacetylase activity H3-K9 specific	2.086e-28	*MBD3*, ***HDAC4***, ***HDAC5***, ***HDAC2***, ***HDAC3***, *HDAC10*, ***HDAC1***, *HDAC11*, *CHD4*, ***HDAC8***, ***HDAC9***, ***HDAC6***, ***SAP30***, ***RBBP4***, ***SIN3A***, ***SAP18***, *RBBP7*
Jensen DISEASES	Intellectual disability	0.1541	*MECP2*, ***HDAC4***, *DNMT3A*, ***HDAC8***, *ACTB*
TargetScan microRNA	hsa-miRNA-3618	0.1148	*MECP2*, ***HDAC4***, ***HDAC2***, ***HDAC3***, *MBD2*, *DNMT3B*, ***SAP18***, *GAPDH*, ***HDAC9***, *TRDMT1*
miRTarBase	hsa-miRNA-92a-3p	0.1033	*MECP2*, *DNMT1*, ***HDAC2***, ***HDAC1***, ***SIN3A***, *MBD2*, *RBBP7*, *GAPDH*, *ACTB*, ***SAP30***
TargetScan microRNA	hsa-miRNA-4536	0.09676	*MECP2*, ***HDAC4***, ***HDAC5***, ***HDAC2***, *DNMT3A*, *DNMT3B*, *HDAC7*
Disease signatures from GEO down 2014	Schizophrenia	0.03222	***HDAC3***, ***RBBP4***, *RPLP0*, ***SAP18***

Enrichr available at: http://amp.pharm.mssm.edu/Enrichr/, accessed on: March 2018. Bold typeset denotes selected genes; p-value threshold was selected as $p < 5 \times 10^{-2}$, significant p-values are highlighted above.

A total of 12 candidate genes from the TaqMan® microarray were selected for further inclusion. The genes and relevant genomic locations are shown below (Table 3.2).

Table 3.2 Candidate genes prioritized

Gene	Chr	GRCh37.p13** location
HDAC1	1	32757708..32799227
HDAC2	6	114257320..114292359
HDAC3	5	141000443..141016423
HDAC4	2	239969864..240323346
HDAC5	17	42154121..42201014
HDAC6	X	48660086..48683408
HDAC8	X	71549366..71792953
HDAC9	7	18126572..19039135
SAP18	13	21714653..21723224
SAP30	4	174292093..174298683
SIN3A	15	75661720..75748181
RBBP4	1	33116749..33151812

*Chr: chromosome; **GRCh37.p13: Human Genome Assembly version 37

3.2. (M1) Variant identification

In summary, the candidate approach resulted in 12 of the TaqMan® Microarray genes being prioritized for inclusion in this study, with a total of 326 known variants identified, 12 of which were annotated with genotype data for the FES cohort. These variants were further prioritized by MAF ≥0.05 for inclusion and further downstream association analyses, this threshold was chosen to include commonly occurring variants in our cohort. A resultant six SNPs were considered for further analyses; rs16835131, rs895808, rs352068, rs17348528, rs3088071 and rs375171. The SNPs were identified in four histone deacetylase (*HDAC*) genes, the Sin3A associated protein (*SAP*) gene, the retinoblastoma binding protein four (*RBBP4*) gene and the syncoilin (*SYNC*) intermediate filament protein gene (Table 3.3).

Table 3.3 Summary of relevant genetic variants

dbSNP ID	Gene	Location (chr;bp)[a]	Alleles		Region
			A1	A2	
rs16835131	*RBBP4 SYNC*	1;33148935	*A*	*G*	3'-UTR, intronic
rs895808	*HDAC4*	2;239970360	*G*	*T*	3'-UTR
rs352068	*HDAC2*	6;114261852	*G*	*A*	Non-coding
rs17348528	*HDAC9*	7;18706703	*C*	*T*	Intronic
rs3088071	*SAP18*	13;21722557	*G*	*A*	3'-UTR
rs375171	*HDAC5*	17;42154470	*C*	*T*	3'-UTR

[a]chr;bp = chromosome: base pair; A1: alternate allele ; A2: ancestral allele ; UTR: untranslated region

The selected variants were investigated with PolymiRTs for their predicted miRNA-related impact and were annotated as function class C, D, or N (Table 3.4). The respective classes annotate variants as follows, C; the derived allele creates a new miRNA binding site, D; the derived allele disrupts a conserved miRNA site, and N; the derived allele disrupts a non-conserved miRNA site. Although some predictions are of variants with effect in non-conserved sites (N) and hence have lesser weighted impact in terms of effect, these were the only predictions and annotation available as identified with this bioinformatic platform. The highlighted miRNA, hsa-miR-130a-5p, overlapped between M1 and M2, as it an input miRNA as identified previously (Hauberg et al., 2016a).

Table 3.4 PolymiRTs prediction output and identification of variants

PolymiRTs SNP ID	Gene ID	miR ID*	Function Class*
rs16835131	*RBBP4*		
	SYNC	hsa-miR-7974	D
rs895808	*HDAC4*	hsa-miR-548ac	D
		hsa-miR-548d-3p	D
		hsa-miR-548h-3p	D
		hsa-miR-548z	D
rs352068	*HDAC2*	**hsa-miR-130a-5p**	C
		hsa-miR-23a-3p	C
		hsa-miR-23b-3p	C
		hsa-miR-23c	C
		hsa-miR-1279	D
		hsa-miR-5007-3p	D
rs17348528	*HDAC9*	hsa-miR-4684-3p	C
rs3088071	*SAP18*	hsa-miR-126-3p	C
		hsa-miR-1206	D
		hsa-miR-452-5p	D
		hsa-miR-4676-3p	D
		hsa-miR-6853-3p	D
		hsa-miR-892c-3p	D
rs375171	*HDAC5*	hsa-miR-1270	C
		hsa-miR-4254	C
		hsa-miR-4308	C
		hsa-miR-4683	C
		hsa-miR-620	C
		hsa-miR-4292	D
		hsa-miR-6791-5p	D
		hsa-miR-4450	N
		hsa-miR-4667-5p	N
		hsa-miR-4700-5p	N
		hsa-miR-6852-5p	N
		hsa-miR-6857-5p	N
		hsa-miR-8089	N

*Linked to miRbase. Functional class identifiers; D: the derived allele disrupts a conserved miRNA site (ancestral allele with support >= 2), C: The derived allele creates a new miRNA site, N: The derived allele disrupts a non-conserved miRNA site (ancestral allele with support < 2). Bold typeset indicated miRNA ID, is a shared miRNA from Hauberg et al., (2016). PolymiRTs available at: http://compbio.uthsc.edu/miRSNP/; accessed on: March 2018.

3.3. (M1) Bioinformatic analyses

When annotating variants, RegulomeDB™ assigns a score to each resultant output. A score of 1 to 6 is allocated with descriptions for scores 1 – 3; "likely to affect binding and linked to expression of target gene" and "minimal binding evidence", and a score of 4 – 6 indicating minimal binding evidence (Boyle et al., 2012). All variants displayed minimal to no binding evidence in RegulomeDB™ scoring (Table 3.5). According to rSNPBase, five out of the six variants displayed evidence for proximal regulation and three with evidence of distal regulation

(Guo et al., 2014b). None of the SNPs had evidence towards miRNA regulation however five out of the six displayed evidence with RNA binding protein mediated regulation. Lastly five variants were reported as eQTL by rSNPBase (Guo et al., 2014b). Variants with assigned scores in relation to these predicted regulatory effects, are described below (Table 3.5).

Table 3.5 Functional and regulatory impact of variants

dbSNP ID	Gene	Regulome DB[a]	rSNPBase[b]				
			Proximal regulation	Distal regulation	miRNA regulation	RNA binding protein mediated regulation	eQTL
rs16835131	*RBBP4*	5	No	No	No	Yes	Yes
rs895808	*SYNCHDAC4*	6	Yes	No	No	No	Yes
rs352068	*HDAC2*	No data	Yes	Yes	No	Yes	Yes
rs17348528	*HDAC9*	No data	Yes	No	No	Yes	No
rs3088071	*SAP18*	4	Yes	Yes	No	Yes	Yes
rs375171	*HDAC5*	4	Yes	Yes	No	Yes	Yes

[a]Results range from 1 to 6 with descriptions of "likely to affect binding and linked to expression of target gene" and "minimal binding evidence": 4/5/6 = Minimal binding evidence; [b] yes = involved, and no = not involved in regulation; eQTL = expression quantitative trait loci; miRNA = microRNA. RegulomeDB available at: http://www.regulomedb.org/index; rSNPBase available at: http://rsnp.psych.ac.cn/index.do, both accessed on: May 2019.

SNPnexus was utilized to corroborate functional impact of the identified variants (Dayem Ullah et al., 2018). Predicted protein functional effects are based on the SIFT and PolyPhen predictions (Adzhubei et al., 2010; Kumar et al., 2009), however no results were returned when the database was queried (available at: https://snp-nexus.org/index.html; accessed on May 2018).

Allelic frequencies were compared to Ensembl minor allele frequencies of the African, European, South Asian and East Asian populations and are shown below (Table 3.6). The Hardy-Weinberg Equilibrium (HWE) value for each variant was determined using SNPstats (Sole et al., 2006).

Table 3.6 Comparative allele frequencies of variants

			Allele frequencies[a]					
dbSNP ID	**Gene ID**	**Alleles (A1/A2)**	**MAF[f] (FES cohort)**	**African[b]**	**East Asian[c]**	**European[d]**	**South Asian[e]**	**HW exact test[g] (p-value)**
rs16835131	*RBBP4 SYNC*	*G/A*	0.05825	0.072	0.099	0.104	0.092	1
rs895808	*HDAC4*	*A/C*	0.3398	0.0290	0.303	0.371	0.282	0.27
rs352068	*HDAC2*	*T/C*	0.05825	0.010	0.076	0.156	0.140	0.29
rs17348528	*HDAC9*	*T/C*	0.1165	0.126	0.175	0.100	0.102	0.35
rs3088071	*SAP18*	*A/G*	0.2864	0.225	0.716	0.452	0.422	0.031
rs375171	*HDAC5*	*G/A*	0.3252	0.821	0.782	0.473	0.449	0.37

[a]All alternate (A2) allele frequencies; [b - e] Obtained from Ensembl 1000 Genomes Project Phase 3, available at: https://www.ensembl.org/index.html; [f] MAF: minor allele frequency, obtained using PLINK v1.90, [g]HW = Hardy-Weinberg equilibrium value, as determined by SNPstats available at: https://snpstats.net/start.htm, both databases accessed on: May 2019.

3.4. (M1) Association analyses with ATR

Significant associations between variants and change in PANSS scores (positive, negative, general, and total) over the 12-month time period (adjusted as previously described) were investigated. Where significant findings were noted, mode of inheritance, odds ratios and confidence intervals were calculated (Table 3.7). After Bonferroni correction ($p \leq 1.04 \times 10^{-3}$), one (rs895808) of the six variants survived multiple testing and was found significantly associated with antipsychotic treatment response. Considering the inheritance models used in this study, significant variants were seen to influence ΔPANSS scores over time based on the presence of a particular genotype or allele respectively. Furthermore, this effect mediated by the respective SNP on ΔPANSS score is recorded as a percentage change, displayed with a

corresponding 95% confidence interval.

Table 3.7 Variants trending to significance (p≤5 x10^{-2}) associated with antipsychotic treatment response as per change in PANSS scores over 12 months, considering genotypic and additive modes of inheritance.

Genotypic Model of Inheritance						
dbSNP ID	**Gene**	**PANSS symptom domain**	**p-value***	**Contrast**	**[a]Percentage change p/month**	**95% CI**
rs3088071	*SAP18*	General	0.005	*GA* vs *GG*	-0.07	-0.132 to -0.002
rs375171	*HDAC5*	Negative	0.033	*CT* vs *CC*	-0.002	-0.076 to 0.072
rs895808	*HDAC4*	Negative	0.003	*GT* vs *GG*	0.035	-0.034 to 0.103
			0.001	*GG* vs *TT*	-0.073	-0.195 to 0.049
rs352068	*HDAC2*	Negative	0.022	*GA* vs *GG*	0.04	-0.079 to 0.150
			0.011	*GG* vs *AA*	-0.42	-0.770 to -0.064
Additive Model of Inheritance						
dbSNP ID	**Gene**	**PANSS symptom domain**	**p-value***	**Contrast**	**[a]Percentage change p/month**	**95% CI**
rs895808	*HDAC4*	Negative	**0.000146**	each *T*	-0.007	-0.060 to 0.046
rs352068	*HDAC2*	Negative	0.00129	each *A*	-0.033	-0.134 to 0.068
		Total	0.0480	each *A*	-0.014	-0.100 to 0.072

*p-value significance threshold (p≤0.05); bold typeset indicates variants passing original Bonferroni correction threshold; [a]Indicates % change in PANSS per month; PANSS = Positive and Negative Syndrome Scale, CI = Confidence Interval

The majority of associations were noted in the negative PANSS domain under both genotypic and additive models of inheritance. One other association was determined in both the general and total PANSS domains, and none were found with significant effect of the positive symptom domain. Overall, all four variants (rs3088071, rs375171, rs895808, and rs352068) were associated with an improved treatment outcome (percentage change less than zero), although interestingly two variants, rs895808 and rs352068, were shown to be significantly associated with a worsened treatment outcome for the contrasting homozygous (*GG*) genotypes seen. When considering the additive mode of inheritance only two variants (rs895808 and rs352068) were found significantly associated with an improved treatment response (percentage change less than zero). Under the genotypic model the largest difference in percentage change between contrasting genotypes was seen for *HDAC2* rs352068, which showed -0.42% change per month ($p = 1.1 \times 10^{-2}$) with the *GG* genotype (versus *AA*), indicative of the greatest improved response to treatment associated with a genotype. In contrast, the largest percent change value conferring

a worsened treatment response associated with a genotype, was observed for this same gene *HDAC2* rs352068, which showed a 0.04% change per month ($p = 2.2 \times 10^{-2}$) for the alternate *GA* heterozygous genotype.

Considering correction for multiple testing only one variant survived (rs895808) and was identified to have a significant association. This association was identified in *HDAC4* and was determined to be significantly associated ($p = 1.46 \times 10^{-4}$) with an improved treatment response indicated by the -0.007% change for the *T* allele.

3.5. (M1) Bioinformatic pathway analysis – NetworkAnalyst

The subnetwork (subnetwork 2) denoted below refers to the interactions observed between the *HDAC4* and *HDAC2* genes. Visual representation of this subnetwork in gene-miRNA interactions are also displayed below (Figure 10). The nature of the microarray from which these genes were selected implies regulatory relevance and likely inclusion or influence of said processes. However, in context of this study the results reported below are representing the most relevant pathways with regard to miRNA-interaction or involvement (Table 3.8 – 3.13).

Table 3.8 Gene-miRNA interaction for biological function

Pathway	p-value
Chromatin remodeling	1.79×10^{-4}
Chromatin modification	3.75×10^{-3}
Regulation of transcription – DNA dependent	1.84×10^{-2}
Regulation of RNA metabolic process	2.04×10^{-2}
Gene silencing	2.06×10^{-2}
Regulation of gene expression – epigenetic	3.22×10^{-2}
Regulation of transcription from RNA polymerase II promotor	3.51×10^{-2}
Notch signaling pathway	3.67×10^{-2}
Transcription from RNA polymerase II promotor	4.97×10^{-2}

Table 3.9 Gene-miRNA interaction for biological function (subnetwork 2)

Pathway	p-value
Negative regulation of RNA metabolic process	5.13×10^{-3}
Regulation of protein modification process	7.62×10^{-3}
Positive regulation of RNA metabolic process	8.7×10^{-3}
Gene silencing	1.38×10^{-2}
Calcium-mediated signaling	1.49×10^{-2}
Regulation of gene expression – epigenetic	2.16×10^{-2}
Regulation of molecular function	2.47×10^{-2}
Response to drug	4.76×10^{-2}

Table 3.10 Gene-miRNA interaction for molecular function

Pathway	p-value
Transcription corepressor activity	1.21×10^{-3}
Transcription cofactor activity	8.27×10^{-3}

Table 3.11 Gene-miRNA interaction for molecular function (subnetwork 2)

Pathway	p-value
Transcription factor binding	1.23×10^{-3}
Negative regulation of transcription – DNA dependent	4.64×10^{-3}
Positive regulation of transcription – DNA dependent	7.54×10^{-3}
Transcription corepressor activity	2.85×10^{-2}

Table 3.12 Transcription factor-miRNA coregulatory network for biological function

Pathway	p-value
Alcoholism	3.57×10^{-10}
Notch signaling pathway	4.44×10^{-9}
Thyroid hormone signaling pathway	1.23×10^{-8}
MAPK signaling pathway	2.19×10^{-4}
Neurotrophin signaling pathway	5.78×10^{-4}
Huntington's disease	2.31×10^{-3}
Cocaine addiction	2.85×10^{-3}
Wnt signaling pathway	3×10^{-3}
Long-term potentiation	8.74×10^{-3}
Amphetamine addiction	9.2×10^{-3}

RNA transport	$5x10^{-2}$

Table 3.13 Transcription factor-miRNA coregulatory network for molecular function

Pathway	p-value
Negative regulation of transcription – DNA dependent	$1.6x10^{-58}$
Transcription corepressor activity	$1.67x10^{-23}$

Figure 3.1 Expected gene-miRNA interaction network for subnetwork 2 as determined by KEGG, for additional miRNA targeting *HDAC2* and *HDAC4*, as well as any miRNAs shared.

3.6. (M2) MiRNA gene target identification

The miRNA-target prediction yielded 4536 target genes, reduced to 1515 genes once duplicates were removed.

3.7. (M2) MiRNA target gene variant identification

Within the above targets ~65200 known variants were identified, 525 of which are genotyped and annotated within the genome-wide genotype data for the FES cohort, and of those 435 presenting with a MAF ≥ 0.05, this threshold was chosen to include commonly occurring variants in our cohort.

3.8. (M2) Bioinformatic pathway analysis

The following table (Table 3.14) displays the results of the gene set enrichment done in Enrichr, alongside the chosen databases and pathways of interest. Categories within respective databases were selected based on both p-value significance and with relevance to biological processes previously linked to SZ and those involving miRNA mechanisms.

The FUMA analyses involved the 535 SNPs as input, all SNPs were annotated with a GWAS significant p-value ($p < 5 \times 10^{-8}$) with a sample size of 50000. As the annotation of the SNPs is the only value of interest in context of this study, these parameters are negligible for accuracy. Variants from a respective reference panel for LD analysis were not included in the analysis. The gene mapping result from the input SNPs is shown below (Figure 3.2) with functional consequences of SNPs on genes (Figure 3.3).

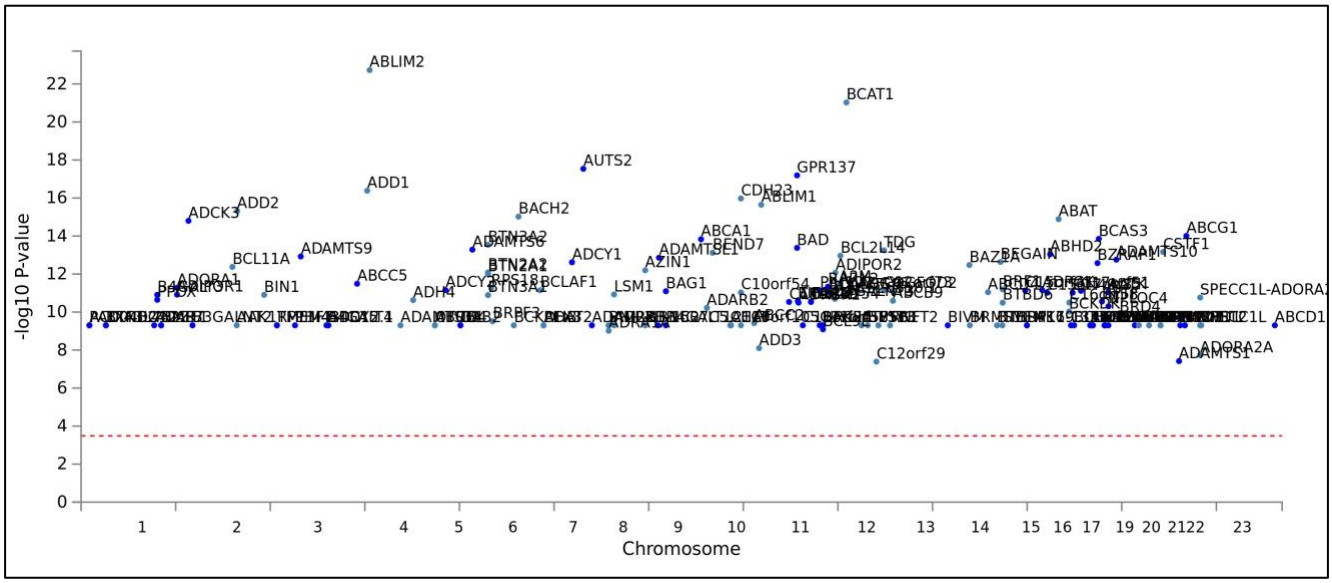

Figure 3.2 Manhattan plot of gene-based test, for 154 genes mapped.

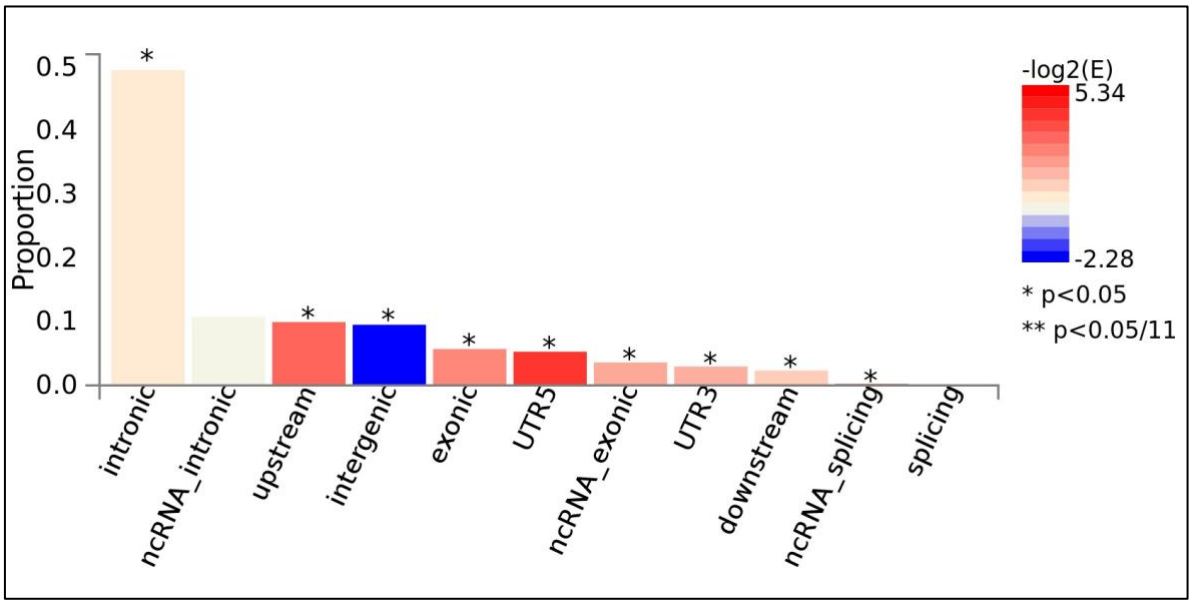

Figure 3.3 Functional consequences of SNPs on genes

The GENE2FUNC results were investigated in WikiPathways (Kelder et al., 2009) and all canonical pathways, also shown below (Figures 3.4 and 3.5). Any results from the GWAS catalog were reported as well (Figure 3.6).

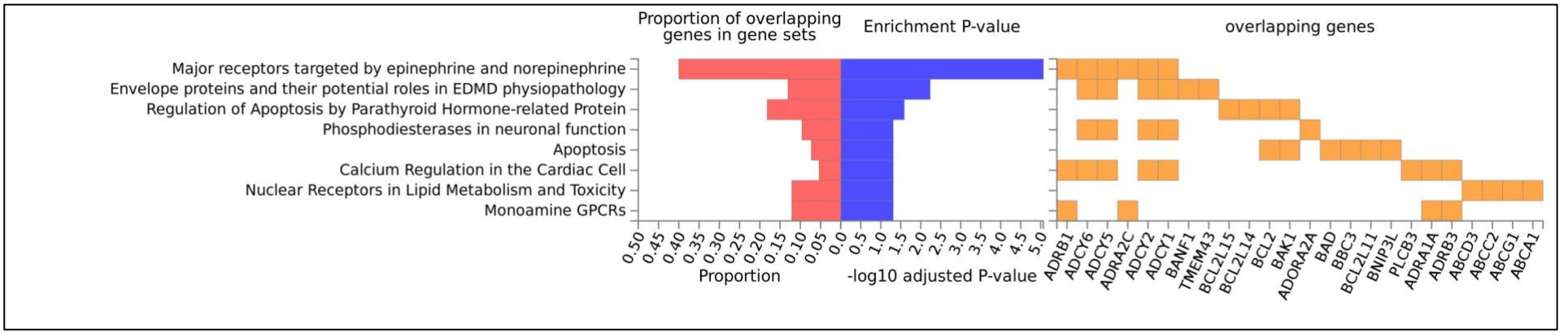

Figure 3.4 WikiPathways results for GENE2FUNC

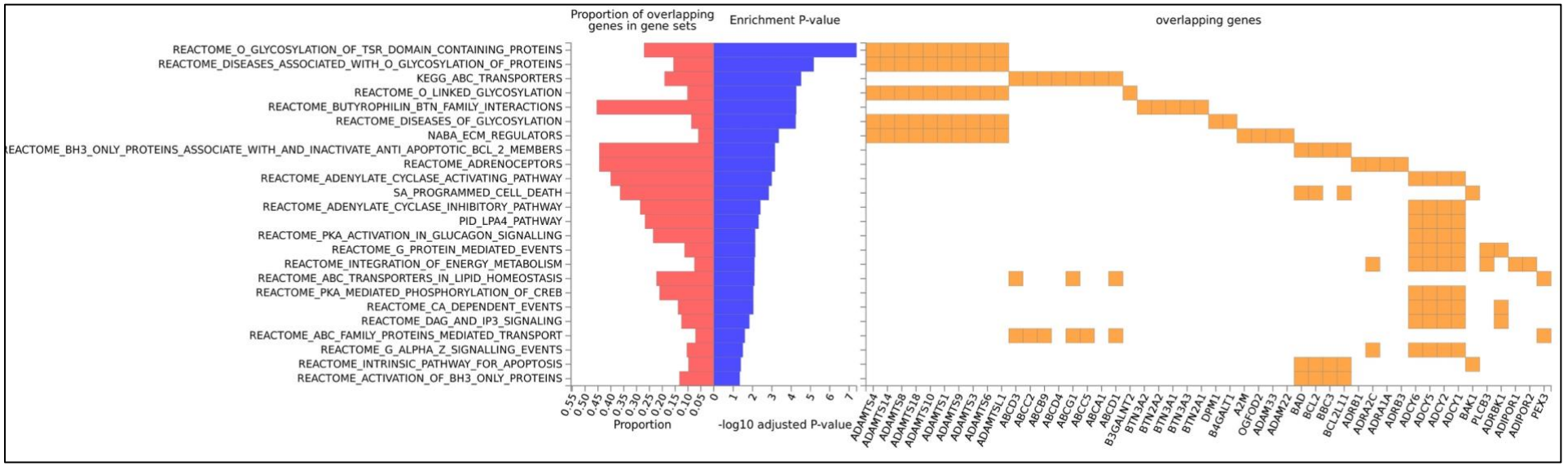

Figure 3.5 All Canonical Pathways results for GENE2FUNC

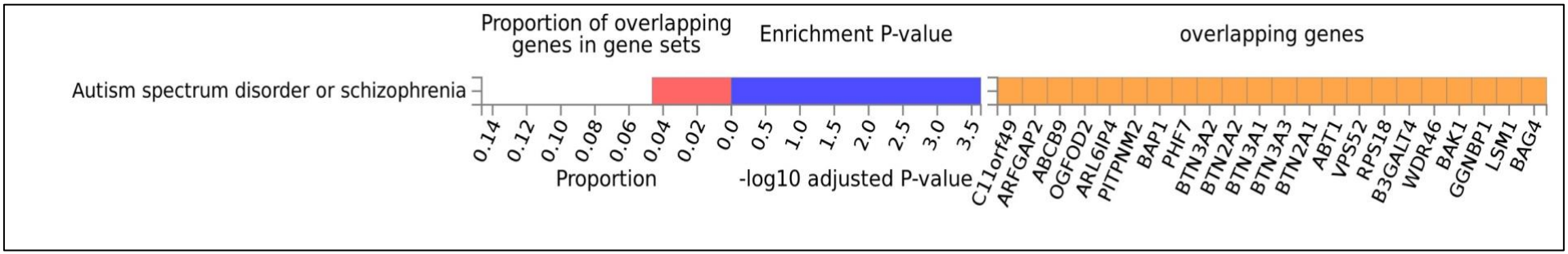

Figure 3.6 GWAS catalog reported genes.

Table 3.14 Gene enrichment categories for miRNA target genes as defined by Enrichr, for unique genes

Gene Set Enrichment Analysis			
Database	**Category/Target/Pathway**	**p-value**	***Gene ID**
Reactome 2016	Gene Expression	2.061e-19	*VARS, TP53RK, EHMT1, NOC2L, C14ORF166, RRP8, TSEN54, TRIM28, RNF111, CSNK1D, KARS, RRAGD, TFAM, TARS2, ZSCAN25, ZNF398, SET, AARS2, BAZ2A, PRDX1, WBSCR22, ZNF264, ZNF383, HARS2, NR2F6, MOV10, AGO4, AGO1, INTS5, INTS6, FARSA, RPS23, EZH2, AARS, BTG2, SMG1, CDKN1B, SRPR, NAT10, MED15, HIST2H2AC, HNF4A, TNKS1BP1, RIOK2, COX8A, PRMT5, PARP1, TET2, DIEXF, HIST2H2BE, AEBP2, CD44, SRRT, RPS27L, COX5A, ZNF226, BRF2, HIPK1, GATAD2B, SKI, MARS, PRPF31, ZNF354A, ZNF354B, ZNF692, CCNT2, RNU1-1, GTF2B, CASC3, APEH, SESN2, ZNF202, SUPT16H, RPS9, DIMT1, NRBP1, HNRNPUL1, GEMIN4, HIST1H2BB, ZNF792, DNMT1, PLD6, ZMAT5, ZNF664, CPSF7, SMURF2, NR4A1, CNOT11, NABP2, MDM2, PIN1, PHF19, DYRK2, DDX47, CHD9, WDR4, ADAR, CHD4, ZNF529, UBTF, ZNF385A, TGIF1, ELAC2, PPM1A, NUP50, BIRC5, ETF1, GTF3C1, GTF3C3, HIST1H2BJ, POLDIP3, BMS1, IGF2BP3, HNRNPA0, HNRNPA3, ARID3A, CARM1, POLR3H, POLR3K, TRMT61A, TNRC6B*
	Translation	7.979e-9	*SRPR, APEH*
	Disease	1.235e-6	*GTF2B, IPO5, RCC1, KPNA5, AP2M1, SUPT16H, AP1B1, ADAM10, VCP, CUL5, CTBP1, BAG4, JAG2, NR4A1, FGFR1, FEN1, HSP90AB1, MTR, HEY1, AP1G1, DLAT, AXIN1, PPP2R5A, VPS37B, PACS1, CANX, AKT1S1, CHMP7, AMER1, FURIN, ERBB3, SYVN1*
WikiPathways 2016	mRNA processing	1.117e-5	*PPM1G, DHX15, RBM39, NONO, HNRNPAB, SUGP2*
NCI-Nature 2016	Regulation of telomerase	2.840e-3	*IFNAR2, PTGES3, SP3*
	ATR signalling	2.840e-3	*TIPIN, TIMELESS*
WikiPathways 2016	Translation factors	4.215e-3	*CLUH*
KEGG 2016	RNA degradation	9.787e-3	*HSPA9, BTG2, BTG1, DIS3L, ENO1, PFKL*
	RNA transport	5.682e-2	*NMD3, CASC3, FXR2, PRMT5, ELAC2, GEMIN4, ACIN1*
WikiPathways 2016	BDNF signaling	1.124e-1	*EGR1, KIDINS220, RANBP9, CFL1*
HumanCyc 2016	Superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis	1.651e-1	*RRM2, CMPK1, NME3, NME4, CTPS1, TYMS*
KEGG 2016	Transcriptional misregulation in cancer	1.655e-1	*ZBTB17, TAF15, KMT2A, MLLT1, HOXA11, ELK4, PER2, MEIS1, EWSR1, ID2, HIST1H3G, WHSC1, HIST1H3B, BCL2L1*
	Thyroid hormone signaling pathway	1.903e-1	*ITGB3, SLC2A1, ATP2A2, ATP1A1, MED12L, BMP4, ITGAV*
NCI-Nature 2016	Signalling events mediated by HDAC class III	2.345e-1	*TUBA1B, TUBB2A, HIST4H4*
	Neurotrophic factor-mediated Trk receptor signaling	3.147e-1	*DNAJA3*
BioCarta 2016	IGF-1 signaling pathway	5.477e-1	*SRF*
NCI-Nature 2016	Wnt signaling network	8.558e-1	*FZD7*

4. DISCUSSION

The role of miRNA-mediated regulation and miRNA targeted genes associated with SZ, in antipsychotic treatment response, has elucidated to underlying mechanisms contributing to the genetics of this trait, including regulatory homeostasis. The novel bioinformatic approach presented here provides the predicted regulatory impacts of variants and a picture of regulatory potential both of the identified variants as well as the mechanisms (*i.e.* miRNAs) involved.

The global reach of miRNA-mediated mechanisms became clear upon the identification of an abundance of miRNA targets (~4k) and numerous SNPs in either method, amassing ~65k variants identified in miRNA target genes alone. Furthermore, in the investigation of variants influencing miRNA-binding/target sites in M1, an overlapping miRNA, hsa-miRNA-130a-5p, was found in the SZ working group of the PGC (Hauberg et al., 2016b). A variant within the *HDAC2* gene was shown to create a binding site for this miRNA. The importance of considering overarching mechanisms in biological systems can be highlighted by the vast number of affected genes revealed in these analyses, as they represent potential feedback loops with regards to regulation and its downstream consequences. The involvement of such systems or pathways may underly mechanisms commonly overlooked, ignored or unknown in complex disorders and associated factors like ATR.

The SNP, rs16835131 was identified occurring in the intronic region of the *SYNC* gene and in the 3'-UTR of *RBBP4*. The syncoilin intermediate filament protein gene (*SYNC*) is a member of the intermediate filament family and is found at neuromuscular junctions, sarcolemma, and Z-lines and thought to be important for muscle fiber integrity (Poon et al., 2002). The chromatin remodeling factor, RBBP4 gene has been described as a core component of a heterochromatin complex, Polycomb Repressive Complex 2 (PRC2), which is responsible for di- and tri-methylation of lysine 27 in histone H3 (H3K27me2/3) (Sudhalkar et al., 2018). Neither of these two genes appear in any literature associated with SZ, and the variant implicated appears to be novel in the context of SZ. Upon identification of this variant, the following miRNA was found to be affected; hsa-miR-7974, the conserved site is predicted to be disrupted by the SNP, rs16835131, when the *G* allele is considered.

The SNP, rs352068 was identified within the noncoding region of the histone deacetylase gene, *HDAC2*, that belongs to Class I of the HDAC gene family. This family is divided by sequence homology into Class I HDACs (1, 2, 3, and 8) and Class IIa HDACs (4, 5, 7, and 9). Previous research has shown that the serotonin 5-HT$_{2A}$ and other monoaminergic receptors are heavily implicated in mechanisms surrounding ATR, with atypical antipsychotics having high affinity binding for such receptors (Wang et al., 2018). Another system largely implicated by such mechanisms is that of the glutamatergic system and the metabotropic glutamate 2 receptor (mGlu2). This receptor has been identified to have significant impact on cellular signaling, and electrophysiological and behavioural functioning which require 5-HT$_{2A}$ expression in cortical pyramidal neurons (Moreno et al., 2009). These two receptors have been seen to alter signaling pathways exerting opposite effects on psychosis and antipsychotic-like behaviors. Atypical drugs were seen to affect 5-HT$_{2A}$ receptor density with further implication for the associated antipsychotic effects, and opposingly induced repressive histone modifications at the promotor region of the mGlu2 gene (*GRM2*) (Kurita et al., 2012). The interactive findings of the 5-HT$_{2A}$ and mGlu2 receptors were described as 5-HT$_{2A}$ receptor-dependent modulation of promotor activity of *HDAC2*, leading to the proposal of inhibition of this gene as a likely therapeutic candidate for ATR efficacy mechanisms. The variant has been identified previously in schizophrenic patients, albeit of Han Chinese ancestry and not presenting with genome-wide significance in association analyses with SZ (Chen et al., 2015). The following miRNAs were found to create a new site when considering the *G* allele of rs352068; hsa-miR-130a-5p, hsa-miR-23a-3p, hsa-miR-23b-3p, hsa-miR-23c, while the *A* allele of the SNP disrupts conserved sites of miRNAs; hsa-miR-1279, and hsa-miR-5007-3p. Interestingly hsa-miR-130a-5p was identified by the PGC-SZ working group investigation as occurring within a SZ risk locus (Hauberg et al., 2016a).

The intronic SNP, rs17348528 was identified in a Class IIa, HDAC gene, *HDAC9*. Schizophrenia-specific deletions were observed in this gene and further implicated HDACs in SZ pathophysiology (Tam et al., 2010). It was also found that decreased expression of *HDAC9* was correlated to increased occurrence of neuronal apoptosis (Morrison et al., 2006), and deletion of the gene was also found in association with a SZ mouse model (Lang et al., 2012a), and in adult post-mitotic neurons, with links to SZ (Lang et al., 2012b). Further literature identified the variant, rs76872642 within *HDAC9* associated with reasoning/problem solving (Nakahara et al., 2018). Despite these lines of evidence, research on *HDAC9* expression in the brain have contradicting results. High gene levels were identified upon initial gene cloning (Zhou et al., 2001), versus relatively low levels found expressed in rat brain tissue (Broide et al., 2007a), and again high expression of *HDAC9a* and *HDAC9b* found in normal brain tissue (Lucio-Eterovic et al., 2008). These inconclusive findings highlight the need for clarification of both expression and functioning of *HDAC9* in the brain and implicated disorders

like SZ. Furthermore, when considering the mechanism of action of HDAC genes in relation to ATR, HDAC9 is of great importance as two variants (rs1178119 and rs11764843) were associated with poorer ATR outcomes (O'Connell et al., 2018). The *C* allele of the variant created a new miRNA binding/target site for hsa-miR-4684-3p.

The 3'-UTR SNP rs3088071 was identified in the *SAP18* gene. The *SAP18* gene encodes the histone deacetylase complex subunit *SAP18* – a component of the *SIN*3-repressing complex involved in enhancement of *SIN3-HDAC1* mediated transcriptional repression, as it is known to be a part of *SIN3* histone deacetylase (*SIN3-HDAC*) complex (Deka and Singh, 2017). This gene also forms part of the apoptosis-and splicing-associated protein (ASAP) complex, known to be imperative for RNA metabolism and hence it may mediate gene expression to an extent (Deka and Singh, 2017). The involvement of *SAP18* in these complexes alludes to the repressive nature of this gene in transcription, achieved in combination with the action of histone deacetylase, and is therefore also recognized for its involvement in deacetylation of histones (Deka and Singh, 2017; Zhang et al., 1998, 1997). Furthermore *SAP18* was identified to have decreased expression when comparing a mouse model for 22q11 deletion syndrome (22q11DS) versus a control (Df1/+ vs wild-type), although *SAP18* was found to be outside the Df1 deletion region (Jurata et al., 2006). Those afflicted by 22q11DS are also known to suffer from SZ, with around 25% of 22q11DS sufferers being affected. Furthermore, the Df1/+ mouse displayed deficits in cognitive functioning mirroring those of SZ symptoms (Bassett et al., 2005; Murphy et al., 2000; Jurata et al., 2006). Identification of the activity-dependent neuroprotective protein (*ADNP*) uncovered its crucial role in neural tube closure and brain formation and further evidence provided by Schirer *et al.*, revealed an interaction of *ADNP* with RNA splicing machinery (Pinhasov et al., 2003; Schirer et al., 2014). Upon investigation of proteins interacting with *ADNP*, *SAP18* was found as an auxiliary component of the splicing-dependent multiprotein exon junction complex (EJC) (Gozes, 2018; Havugimana et al., 2012). This complex is found deposited at the splice junction on mRNAs and provides potential evidence of this interaction between *SAP18* and *ADNP* via RNA splicing machinery and hence implicates *SAP18* in proper cognitive development and functioning. The *A* allele consequentially created a new binding/target site for hsa-miR-126-3p and disrupted conserved sites for hsa-miR-1206, hsa-miR-452-5p, hsa-miR-4676-3p, hsa-miR-6853-3p, and hsa-miR-892c-3p.

The 3'-UTR SNP, rs375171 occurs in another histone deacetylase gene, *HDAC5* which is known for critical roles in neuronal plasticity, survival and differentiation and binds to and represses myogenic transcription factors of the myocyte enhancer factor-2 (MEF-2) (Chawla et al., 2003). This gene was found to be differentially expressed when comparing SZ-subtypes (chronic vs FES vs controls) with

significant association with ATR in a South African cohort (O'Connell et al., 2019). However, this finding was reported for a different variant, rs11079983, with increased expression and in association with a poorer treatment outcome for the *TT* genotype (versus *CC*) when considering the PANSS-negative domain (O'Connell et al., 2019). Concluding the findings of their differential expression analyses, the authors discussed the potential alteration of specific pathways, resultant of altered gene expression in reducing the expression of *ASB16* and increasing *ASB16-AS1* in the cerebellum. Such pathways are the ubiquitin-mediated and cytokine signaling pathways, and these results were noted with the *TT* genotype of rs11079983, with a suspected advantage for ATR (Kohroki et al., 2005; Babon et al., 2009). The identification of the variant rs375171 is accompanied by a host of predicted miRNA consequences (Table 3). New binding site creation and disruption of a conserved site being most notable considering the sensitivity of histone deacetylase genes to environmental influences themselves. This SNP *T* allele was predicted to create a new miRNA binding/target site for hsa-miR-1270, hsa-miR-4254, hsa-miR-4308, hsa-miR-4683, and hsa-miR-620, whilst the *C* allele disrupts the conserved sites of hsa-miR-4292 and hsa-miR-6791-5p, and finally to disrupt of non-conserved sites for hsa-miR-4450, hsa-miR-4667-5p, hsa-miR-4700-5p, hsa-miR-6852-5p, hsa-miR-6857-5p, and hsa-miR-8089.

Whilst there were a multitude of miRNAs identified, those of most relevance pertain to having creation of a new miRNA site or disruption of a conserved site by interaction of the variant identified, for the context of this study the significant SNP associated with ATR outcomes and the respective miRNAs will be discussed in detail.

The only association that survived Bonferroni correction in the association analyses was in the 3'-UTR of the *HDAC4* gene. The SNP rs895808 was significantly associated with an improvement in treatment response when considering both the additive and genotypic mode of inheritance ($p=1x10^{-3}$ and $p=14.6x10^{-6}$ respectively) and was observed as a reduction in PANSS negative scores. This gene amongst others is a member HDAC gene family, which are epigenetic regulators responsible for removing acetyl groups for DNA-histone compaction in regulation of transcription mechanisms (Weïwer et al., 2013b). Belonging to Class IIa, *HDAC4* displays tissue specificity and translocation between the nucleus and cytoplasm, and has been shown associated with SZ in an Asian ancestry cohort (Kim et al., 2010). *HDAC4* was shown amongst others, in a rat with varied expression across all major brain regions and was amongst the highest expressed HDACs (Broide et al., 2007b). This gene notably does not have a mechanism by which binding to DNA can occur directly, but rather interacts with transcription factors *MEF2C* and *MEF2D* to do so (Weïwer et al., 2013b). No evidence for association of the SNP, rs895808, with SZ could be identified in literature. *HDAC4* is thought to

be involved in mediating neuronal apoptosis in cerebellar granule neurons (Bolger and Yao, 2005). As with all cells that function optimally, neurons must maintain a balance between synthesis and degradation of proteins (Schneider et al., 2017). The *G* allele variant disrupts conserved sites of hsa-miR-548ac, hsa-miR-548d-3p, hsa-miR-548h-3p, and hsa-miR-548z, as well as having been linked to miRNA-4536. The disruption of the miRNA site may alter binding and consequentially affect gene expression of *HDAC4* and other genes targeted by the miRNAs, with potential impact on crucial cellular functions. Given that the association found was regarding the negative symptom domain, a regulatory alteration may impact this alleviation in ATR.

While the miRNA-548 family hasn't been linked to schizophrenia in functional implications, the characterization of this miRNA family has been met with many difficulties in that this specific family evolved from a transposable element (Piriyapongsa and Jordan, 2007), and thus members of this family consequentially overlap in functionality and are likely to interact with one another (Liang et al., 2012). However the microRNA, miRNA-548ac has been uncovered with interesting findings in multiple sclerosis (Hecker et al., 2019). Hecker *et al*., (2019) identified putative gene targets of miRNA-548ac, including *DNAJC3* and *HERPUD1*, which are known to promote inflammatory responses and/or the folding and degradation of proteins. Neuroinflammation has been well established in the pathophysiology of schizophrenia, with pro-inflammatory cytokines reported in immunological findings (Smith and Maes, 1995), this presents a potential avenue for underlying miRNA-mediated regulatory effects to be investigated in treatment response of SZ. The additional miRNAs of the miRNA-548 family listed above returned no significant results for functionality in relation to any neurodevelopmental disorders and hence require further investigation in this context. Further validation would be required to observe these interactions to confirm hypotheses, however dysregulation could impede downstream biological pathways implicated in ATR.

The regions in which the above variation occurred are also important to note, considering potential impact on miRNA-mediated regulation, however, seemingly obvious regions like the 3'-UTR are not the only regions of considerable significance. Genes which may have involvement in miRNA biogenesis and in pri-, pre-, and mature miRNA sequences, and hence variation within them, may also be implicated in this regulatory mechanism (Zhang et al., 2015). The 3'-UTR is of particular interest as variation occurring within a miRNA target site may depict a more pathway-specific effect downstream (Mishra and Bertino, 2009). A disruption in a conserved miRNA site may cause loss of ordinary repression control, which in itself has implications for gene expression mechanisms and potential knock-on effects underlying ATR mechanisms. Disruption to non-conserved sites however, present a difficulty regarding the degree to which these polymorphisms influence global mechanisms

such as miRNA-mediated regulation. The creation of a new miRNA site however could affect the expected gene expression patterns of that targeted mRNA. All the above SNPs, but one (rs16835151), was predicted to influence proximal regulation. None of the SNPs were predicted with miRNA regulation however most (all excluding rs895808) appeared to be predicted with RNA binding-protein mediated regulation. Half the identified SNPs (rs352608, 3088071, and rs376171) had predicted distal regulatory associations and all but one (rs17348528) presented with eQTL evidence.

The pathway analyses revealed concurrent themes of both regulation and the dysregulation of transcription, translation and associated factors, that may be represented by transcriptional homeostasis and dysregulation of transcription respectively. However, the pathway analysis was only considered in interaction with miRNA mechanisms as the original microarray was composed for transcriptional regulation. Many neuropsychiatric disorders like SZ have underlying interneuron transcriptional dysregulation (Lewis and Hashimoto, 2007). During development and learning the brain is composed of neurons that must elicit some plasticity in continuation of normal functioning whilst neurogenesis is occurring, and synaptic connections between the neurons are developing, strengthening, being altered and pruned (Ramocki and Zoghbi, 2008). Therefore, neurons within affected networks are required to make homeostatic responses to re-establish a balance between excitation and inhibition. Clinical studies have shown that loss or gain in dosage of proteins or RNAs in different neurodevelopmental disorders often result in similar or overlapping sets of neurological symptoms, which has been suggestive of molecules involved in cognitive and behavioral processes partaking in highly regulated homeostatic mechanisms (Ramocki and Zoghbi, 2008).

The mechanism of miRNA-mediated regulation provides a potential feedback loop of regulation by which there may be association of these underlying mechanisms in the ATR of SZ. Subnetwork 2 illustrates two ATR associated genes linked to miRNAs implicated in shared and individual regulation of these genes. These miRNAs were additional to those identified in this study however it substantiated how vast the spectrum of miRNAs responsible for a singular gene are and therefore their global reach. The FUMA pathway analysis results identified implicated pathways that are potential avenues for miRNA-mediated regulation as said genes were targeted by known SZ risk loci miRNAs. The WikiPathways results relevant for this study are; the regulation of apoptosis by parathyroid hormone-related protein, apoptosis and phosphodiesterase's in neuronal function. These influences are of importance due to altered apoptosis implicated in abnormal neurodevelopment as well as neurodegenerative processes (Gassó et al., 2014). Synaptic or neuronal deficits may be alluded to an increased susceptibility to apoptosis as seen in SZ patients (Jarskog et al., 2005). Despite this evidence of involvement in SZ pathophysiology, apoptosis is yet to be investigated in context of

ATR, with these results indicating a potential avenue for exploration in the alleviation of ATR. Phosphodiesterase's are key enzymes in cellular signaling pathways, and in reducing cyclic Mono Phosphate (cAMP) and/or cyclic Guanosine Mono Phosphate (cGMP) (Halene and Siegel, 2007). The mechanism of action of currently used antipsychotics is via antagonism of the D2 receptor. Upon the binding of dopamine to a receptor subtype a cascade of events result in a decrease of adenylyl cyclase activity, reducing cAMP formation and activation of Protein Kinase A (PKA) (Halene and Siegel, 2007). PKA activity has been observed to produce therapeutic benefits of AP treatment, with phosphorylation of various intracellular substrates depending heavily on this pathway activation. AP drugs are thought to oppose this action of dopamine at the D2 receptor and increase cAMP levels and downstream effects, indicative of a functional cAMP signal transduction pathway necessary for AP drug action (Burris et al., 2002). In the review compiled by Halene and affiliates (2007), phosphodiesterase inhibitors are highlighted as potential therapeutic targets, this interaction with miRNA-mediated regulation may hold potential for ATR in related pathways as identified. The Canonical Pathways analysis results had some relation to the above WikiPathways, with the intrinsic pathway for apoptosis and PKA activation in glucagon signaling amongst others. AP drugs have been investigated regarding influence on relevant pathways as they can regulate intracellular signaling molecules which in turn regulate signaling pathways (Huang and Song, 2019). The targeting by miRNA offers a mediated regulatory cascade for pathways that may underlie ATR mechanisms and hence offer therapeutic targets.

Finally, this research can contribute to the knowledgebase on African patients. African populations have been underrepresented in neuro- and pharmacogenomics research in the past (Quansah and McGregor, 2018). Despite evidence regarding the clear burden of non-communicable diseases in Africa, of which NP disorders are amongst the most prevalent, an enormous under-representation of African ancestry in large-scale neurogenomic studies remains (Mayosi et al., 2009b; Quansah and McGregor, 2018; Sirugo et al., 2019). As little as 11.1% of 569 neurogenomic studies was found to be representative of African ancestry cases (Quansah and McGregor, 2018). More recently however, the current GWAS catalogue showed a despairingly small representation of ancestry categories with as little as 2% of individuals reported of African ancestry (Figure 16) (Sirugo et al., 2019). The large variation attributed to intra- and inter-population substructure contributes to the identification of associations and potential drug response factors in complex disorders, in African individuals, and needs to be explored with inclusion of cohorts of African ancestry (Campbell and Tishkoff, 2008; Tishkoff et al., 2009a). This variation presents additional problems that consequentially extend to those already faced in targeted therapies and precision medicine development in Africa. Lower socio-economic classes like those found in Africa are limited by both availability of the best treatment

options as well as the options themselves – both in treatment efficacy and potential adverse effects of the available drugs (Campbell and Tishkoff, 2008; Daar et al., 2007; Warnich et al., 2011a).

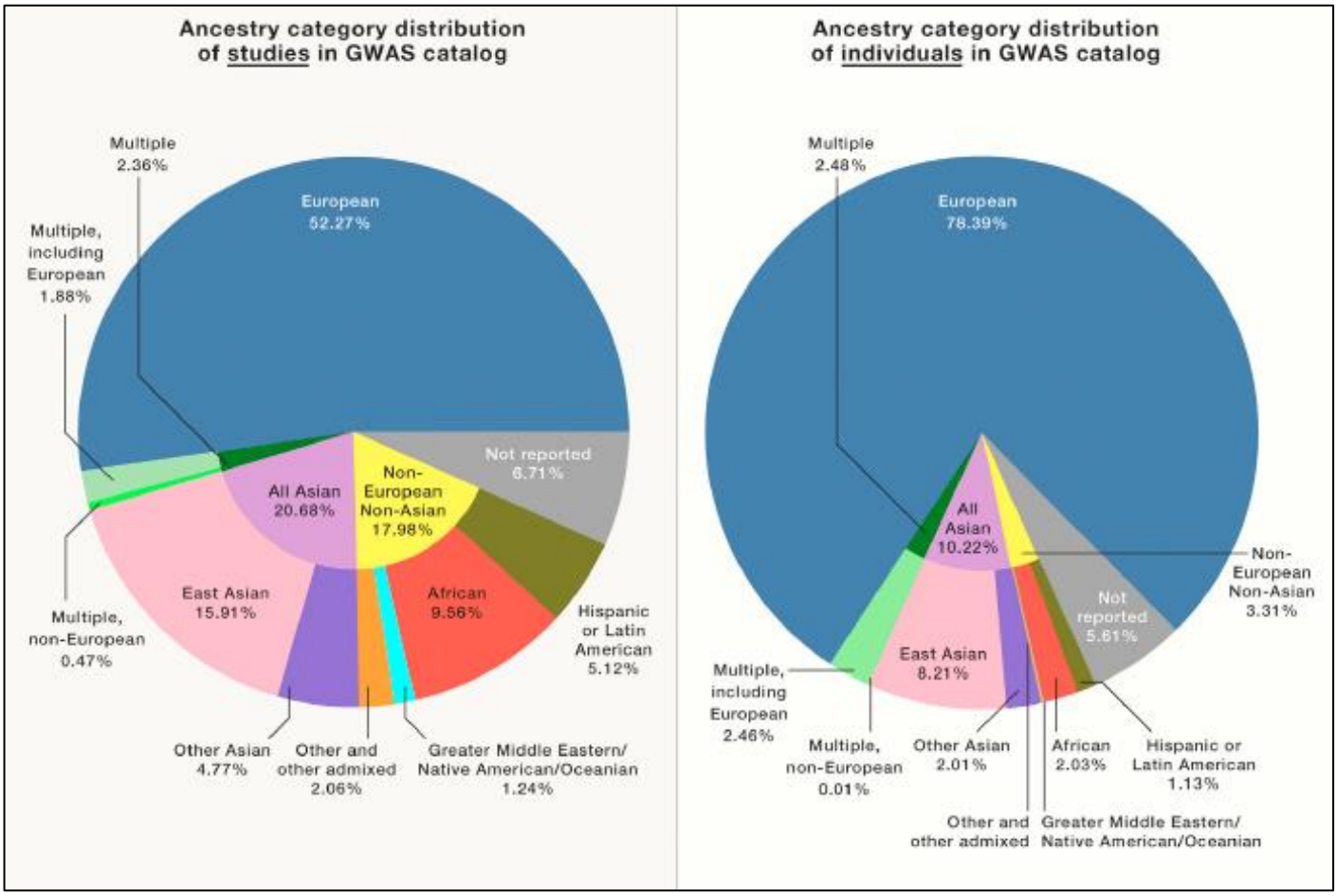

Figure 4.1 GWAS by ancestry for studies in GWAS catalog through January 2019 (Sirugo et al., 2019). *Reprinted with permission from Elsevier*

Moreover the few pharmacogenomic studies that have been performed regarding South African populations have largely been based on candidate genes in relatively small cohorts and focus on different diseases of interest such as HIV and tuberculosis (Warnich et al., 2011b; Chimusa et al., 2013; Swart et al., 2016). With such limited inclusion in large-scale research and the likelihood that GWAS allele frequencies may vary by 20 to 40-fold between population groups (Adeyemo and Rotimi, 2010), resultant outputs may not always be applicable to African populations. All of the above evidence and factors necessitate an important need for further pharmacogenetic research in diverse African, and South African, populations to better understand the underlying mechanisms of NP disorders and to aid in the development of effective treatment, with the aim of lessening the socio-economic burden of such disorders

African populations and their importance in genomics research has been well established. Since these genetically diverse ethnic population groups are vital for fully understanding the genetic basis of NP disorders and respective drug treatments, the exclusion of such populations will likely exacerbate health inequalities (Sirugo et al., 2019). The wide-ranging history of the exploration of southern Africa resulted in varied genomes of the individuals residing there – extending from ancient and genetically diverse, to highly admixed and more recently, homogenous (Warnich et al., 2011a). It is well-known that the age of African populations contributes to their considerable genetic diversity. These populations having been exposed to fewer founder effects, varying diets, climates, infectious diseases, and geography resulted in the highest observed levels of within-population genetic diversity (Cavalli-Sforza, 1997; Campbell and Tishkoff, 2008; Warnich et al., 2011a). As such, individuals of African ancestry constitute the largest genetic variation among global populations. Consequentially, disregarding this variation in genomic research will negatively implicate the identification of potential risk loci and gene associations in complex NP disorders when inclusion of such individuals is limited (Quansah and McGregor, 2018; Warnich et al., 2011a). Given different evolutionary histories, different genetic mutations in the same gene may account for a given disease in diverse populations due to the phenomenon of allelic heterogeneity of complex illnesses (Botstein and Risch, 2003). However variation in causative mutations may thwart said disease diagnoses or subsequent treatment design (Sirugo et al., 2019). The replication of genetic associations across populations can be influenced by several factors, such as linkage disequilibrium (LD). Importantly, markers in LD with risk variants in Europeans may not necessarily be in LD in other, more diverse populations due to LD patterns reflecting differing demographic histories that vary across the globe (Sirugo et al., 2019). These differences in LD across ethnicities impact how well causal variation is captured by tagging SNPs identified in a single population. The “out-of-Africa” origin theory saw modern humans migrate out of Africa some 300k years ago, however African populations have maintained larger and more sub-structured populations and as a result more diverse LD patterns are seen across the continent (Tishkoff et al., 2009b).

African populations are not only important to include in genomics research, but in pharmacogenetics research as well. Notably the antiretrovirals efavirenz (EFV) and rifampicin (RMP) used in HIV/AIDS and tuberculosis treatment were shown to alter miRNA and subsequent drug metabolizing enzyme genes (Swart and Dandara, 2019). Studies like this illustrates the necessity for inclusion of all aspects of pharmacogenetic contributors, as important drug metabolizing genes like *CYP3A4*, *CYP3A5* and *CYP2B6* are amongst those affected. Clinical implications for such findings are paramount, with interindividual differences attributing to drug metabolism and response prediction (Swart et al., 2016).

Warfarin is a commonly prescribed anticoagulant with dosage prescribed based on drug metabolizer genotype (Drozda et al., 2015). However, pharmacogenetic algorithms from trials based on primarily Caucasian and European populations only included specific variants in the vitamin K epoxide reductase complex 1 (*VKORC1*) gene, as well as within the cytochrome P450 2C9 (*CYP2C9*) gene (International Warfarin Pharmacogenetics Consortium, 2009). Importantly these warfarin dosage associated genes account for 40% of the interindividual dosage variability that is seen in the Caucasian population (Takeuchi et al., 2009). When investigating dosage effects of this drug in African populations, a staggering 26 novel *CYP2C9* SNPs and only three previously described *VKORC1* variants were identified in the South African black population (Mitchell et al., 2011). These findings have implications for the major drug metabolizer CYP family enzymes with different allelic combinations resulting in varied enzyme activity (Warnich et al., 2011b). Variation in both these genes, combined with a small subset of environmental factors, were found by Mitchell and colleagues (2011) to contribute to roughly 45% of the heterogeneity in warfarin dosage. The importance of population-specific pharmacogenetics has been reiterated when even a singular nucleotide polymorphism was shown to influence optimal dosage (Ndadza et al., 2019).

Further population-specific diversity of the CYP family was illustrated with the *CYP2D6* gene, an important metabolizer of approximately 20% of all prescribed medication, including antidepressants and antipsychotics (Goldstein, 2001). The highly polymorphic nature of this gene is well known and accounts for variable allele frequencies across different populations (Owen et al., 2009), with reiteration seen in the identification of novel, reduced function, and non-functional alleles across African populations (Dandara et al., 2014, 2001; Masimirembwa et al., 1996). High prevalence of a reduced function allele explained in part the large presence of those individuals classified as intermediate metabolizers in African populations (Masimirembwa et al., 1996; Gaedigk et al., 2008; Matimba et al., 2009), and confirms the uniqueness of African population genomes. The importance of this gene family in drug metabolism therefore has implications for the use of antipsychotics in said populations and these studies collectively illustrate the need for Afrocentric pharmacogenomic research (Dodgen et al., 2017).

With specific regard to antipsychotic pharmacogenetic research, work has been done to better represent the genetic variability seen (Dandara et al., 2001). Drogemöller *et al*., (2014) performed exome sequencing on 11 Mixed-Ancestry (MA) South African FES patients followed by variant prioritization and genotyping of larger admixed FES and Xhosa case/control cohorts, respectively. The resultant identification of multiple loss-of-function variants encapsulated these unique

differences as the majority were either previously unidentified or occurred at dismissably low frequencies in Asian and European population groups (Drogemöller et al., 2014b). The development of the Southern African Human Genome project has also been an important addition to genomics research via characterization of 24 African genomes (Choudhury et al., 2017). Deep whole-genome sequencing as such has identified ~16 million unique variants, with unexplored genomic and pharmacogenetic potential.

5. CONCLUSION

The majority of the above described genes and identified variants occurred in noncoding regions of the genome, and with the majority of predicted miRNA effects occurring as disruptions of conserved sites or creation of new miRNA binding/target sites. Despite the prior knowledge of these genes implicated in regulation, it must be noted that these genes were assessed considering DNA methylation and transcriptional repression specifically, without miRNA-mediated regulatory mechanisms. It is clear that although few genes were selected for this study, multiple miRNAs were predicted to be affected in one way or another by variation in said genes. As suspected and in line with GWAS findings, most variation seen was either in noncoding or within the 3'-untranslated regions which reiterates the importance of said regions in annotation and interpretation of GWAS results.

The association analyses above have identified novel variant associations to antipsychotic treatment outcomes, with the most significant findings in the negative symptom domain – which has been regularly and consistently difficult to treat and have alleviation for schizophrenic patients. The ability of miRNAs to have wide effect on gene expression and functional pathways has shown to have implications in many psychiatric disorders, with schizophrenia characterized by dysregulation of multiple signaling pathways (Miller and Wahlestedt, 2010). The focus on miRNAs and miRNA-mediated regulatory mechanisms in particular have shown an avenue to explain the dysregulation seen in said signaling pathways, with the pathway analyses above highlighting a common trend of transcriptional disruption. Further functional characterization of the miRNAs involved will allow for a more accurate targeting of strategies that can aim to restore whole gene network functionality and homeostatic balance in the context of antipsychotic treatment response. Contextually all miRNAs identified could not be discussed, however the vast amount identified per so few genes reiterated the importance in understanding the underlying biological and molecular functions these dynamic molecules may have.

The hypothesis-free approach in combination with the miRNA-target approach allowed for an identification of both concordance and discordance in the pathway analyses. Although overlap was already determined in the transcriptional capacity, many other interesting pathways were identified in the enrichment analyses that too have been related to schizophrenia previously, like the Wnt, neurotrophin, MAPK, Notch and thyroid hormone signaling pathways. Importantly this study reaffirmed that there are underlying mechanisms capable of interacting and influencing pathways and systems that may influence antipsychotic outcomes in neurodevelopmental disorders. MiRNAs may influence specific pathways and systems that in turn are mediating responses seen in regulatory consequences and furthermore are representative of both the noncoding regions importance in large-scale study designs as well as the environmental influence that persists in neurodevelopmental disorders like schizophrenia. Lastly in incorporating diverse population groups such as that in the FES cohort, adds to the very limited genomic data surrounding African populations, and allows for unique African profiles to be accurately and properly identified and assessed.

5.1. STUDY LIMITATIONS

This study primarily utilized bioinformatic strategies and in absence of RNA-seq data expression analyses were not possible. Therefore, the results that have been shown are predictive and open to further interpretation where possible functional validation would ideally confirm the findings. The ethnicities composed in the cohort presented difficulty in that there is a lack of accurate references available and hence a lack of imputation and a reduced number of SNPs available with genotype information.

5.2. STUDY STRENGTHS

Despite the size of the FES cohort it is however important to note the unique features of the cohort. The cohort is extremely well-characterized and homogenized, with all patients being treatment-naïve at the start of the study. Importantly all participants were administered the same LAI antipsychotic which ensured adherence to treatment. Smaller clinical cohorts of well-characterized individuals have been shown to have equivalent power to cohorts of larger size with less well-characterized patients (Samuels et al., 2009). First-episode cohorts have also shown to provide increased power in pharmacogenetic studies (Zhang and Malhotra, 2013b). With these factors in mind, this study minimized confounders and provided enough statistical power to detect associations (Reynolds, 2007), which was further demonstrated by the surfacing of significant findings surviving multiple testing. Lastly this cohort was beneficial in that few other antipsychotic pharmacogenetic studies exist

with cohorts of similar size, albeit for one study which found and replicated a genome-wide signal in a cohort of comparable size (n = 139) (Malhotra et al., 2012a).

5.3. FUTURE PERSPECTIVES

While the large-scale studies like GWAS and massive consortia of combined efforts in the PGC working groups have amassed and contributed the majority of data and knowledge to the study of neuropsychiatric disorders, these studies do not encompass all aspects of underlying biology. It is most likely a combination of rare, common, and *de novo* variation (van Dongen and Boomsma, 2013), with additional systems genetics influencing implicated gene networks, that resultantly produce traits of such complexity like antipsychotic treatment response. A combined approach that encompasses all such variables, including functional validation studies, within larger GWAS-like cohorts should be the way forward. However, the obvious key is cohort size, even more-so when considering global mechanisms that may unveil more overarching mechanisms underlying complex traits, pharmacogenomic research can only hope to advance with the employment of PGC-comparable cohorts of thousands to tens of thousands of participants, as has already been shown (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

The generation of data from these working groups is massive, however little goes beyond identification of loci, or molecular entities (Hauberg et al., 2016a), the need for thorough interpretation, interrogation and investigation into functional and molecular aspects of such data is apparent by the lack of in vitro research models for antipsychotic treatment response in disorders like schizophrenia. However, there is potential considering the likes of new technologies ability to develop induced pluripotent stem cell (iPSC)-derived neurons, *in vitro* studies of brain morphology under different states are viable. Brennand and associates (2011) differentiated iPSCs into neurons for characterization of schizophrenia. The schizophrenia phenotype neurons showed diminished neuronal connectivity in conjunction with decreased neurite number, PSD95-protein levels, and diminished glutamate receptor expression (Brennand et al., 2011). Further gene expression profiles on the neurons revealed altered expression of components of the cyclic AMP and Wnt signaling pathways, and even more promising a pharmacogenetic finding of amelioration of key cellular and molecular components of the SZ phenotype by Loxapine. The identification of the Wnt signaling pathway may also allude to underlying mechanisms like that of miRNA-mediated regulation considering the results of the current study. However, studies of this nature can confirm predictive tools like PolymiRTs, NetworkAnalyst, and others by validated expression analyses.

APPENDIX

Supplementary Table 1. TaqMan® Human DNA Methylation and Transcriptional Repression microarray genes*

#	Assay IDs	Gene
2	Hs99999905_m1	*GAPDH*
3	Hs99999909_m1	*HPRT1*
4	Hs99999908_m1	*GUSB*
5	Hs99999903_m1	*ACTB*
6	Hs99999907_m1	*B2M*
7	Hs99999902_m1	*RPLP0*
8	Hs00609297_m1	*HMBS*
9	Hs00172349_m1	*CHD4*
10	Hs00154749_m1	*DNMT1*
11	Hs01027166_m1	*DNMT3A*
12	Hs00171876_m1	*DNMT3B*
13	Hs02621185_s1	*HDAC1*
14	Hs00368899_m1	*HDAC10*
15	Hs00227335_m1	*HDAC11*
16	Hs00231032_m1	*HDAC2*
17	Hs00187320_m1	*HDAC3*
18	Hs00195814_m1	*HDAC4*
19	Hs00608366_m1	*HDAC5*
20	Hs00195869_m1	*HDAC6*
21	Hs00248789_m1	*HDAC7*
22	Hs00218503_m1	*HDAC8*
23	Hs00206843_m1	*HDAC9*
24	Hs00969366_m1	*MBD2*
25	Hs00172710_m1	*MBD3*
26	Hs00172845_m1	*MECP2*
27	Hs00428403_g1	*RBBP4*
28	Hs00171476_m1	*RBBP7*
29	Hs00705532_s1	*SAP18*
30	Hs01009154_g1	*SAP30*
31	Hs00411592_m1	*SIN3A*
32	Hs00189402_m1	*TRDMT1*

**Applied Biosystems, California, USA*

Pathways description: Transcriptional repression is an essential mechanism in the precise control of gene expression. Transcriptional repressor proteins associate with their target genes either directly through a DNA-binding domain or indirectly by interacting with other DNA-bound proteins. To inhibit transcription in a selective manner, a repressor protein can (1) mask a transcriptional activation domain, (2) block interaction of an activator with other components of the transcription machinery, or (3) displace an activator from the DNA. Furthermore, DNA response elements can exert allosteric effects on transcriptional regulators, such that regulators may activate transcription in the context of one gene yet repress transcription in another. The most direct mechanism by which DNA methylation can interfere with transcription is to prevent the binding of basal transcriptional machinery or ubiquitous TF (Transcription Factors) that require contact with cytosine (C) in the major groove of the double helix. Transcriptionally active chromatin is predominantly unmethylated and has high

levels of acetylated Histone tails. Most mammalian TFs have GC-rich binding sites, and many have CpGs in their DNA recognition elements. Binding by several of these factors is impeded or abolished by methylation of CpG.

Supplementary Table 2. 43 mature miRNAs identified by PGC-SZ working group

miRNAs in SZ risk loci
miRNA-29b-2-5p
miRNA-29b-3p
miRNA-29c-3p
miRNA-29c-5p
miRNA-33a-3p
miRNA-33a-5p
miRNA-33b-3p
miRNA-33b-5p
miRNA-130a-3p
miRNA-130a-5p
miRNA-137
miRNA-378
miRNA-640
miRNA-1228-3p
miRNA-1228-5p
miRNA-1281
miRNA-1307-3p
miRNA-1307-5p
miRNA-2682-3p
miRNA-2682-5p
miRNA-3160-3p
miRNA-3160-5p
miRNA-3655
miRNA-4301
miRNA-4304
miRNA-4529-3p
miRNA-4529-5p
miRNA-4655-3p
miRNA-4655-5p
miRNA-4677-3p
miRNA-4677-5p
miRNA-4688
miRNA-6773-3p
miRNA-6773-5p
miRNA-6777-3p
miRNA-6777-5p
miRNA-6843-3p
miRNA-6889-3p
miRNA-6889-5p
miRNA-8064
miRNA-8072

(Hauberg et al., 2016a)

Supplementary Tables 3 – 8. Enrichr results of miRNA-target gene enrichment

KEGG 2016 via Enrichr			
Term	P-value	Adjusted p-value	Z score
Cell cycle_Homo_sapiens_hsa04110	1.65×10^{-6}	4.53×10^{-4}	-1.73
Ribosome_Homo sapiens_hsa03010	11.2×10^{-6}	1.55×10^{-4}	-1.72
Viral carcinogenesis_Homo sapiens_hsa05203	17×10^{-5}	9.78×10^{-3}	-1.93
RNA degradation_Homo sapiens_hsa03018	18×10^{-5}	9.78×10^{-3}	-1.74
Spliceosome_Homo sapiens_hsa03040	175×10^{-6}	9.78×10^{-3}	-1.72
RNA transport_Homo sapiens_hsa03013	12.4×10^{-4}	5.7×10^{-2}	-1.74
Proteoglycans in cancer_Homo sapiens_hsa05205	5.88×10^{-3}	1.8×10^{-2}	-1.80
Protein processing in endoplasmic reticulum_Homo sapiens_hsa04141	4.43×10^{-3}	1.65×10^{-2}	-1.67
Prostate cancer_Homo sapiens_hsa05215	7×10^{-3}	1.9×10^{-2}	-1.71
Transcriptional misregulation in cancer_Homo sapiens_hsa05202	5×10^{-3}	1.65×10^{-2}	-1.58
Thyroid hormone signaling pathway_Homo sapiens_hsa04919	7.61×10^{-3}	1.9×10^{-2}	-1.59
Biosynthesis of amino acids_Homo sapiens_hsa01230	9.32×10^{-3}	2.13×10^{-2}	-1.41
Progesterone-mediated oocyte maturation_Homo sapiens_hsa04914	1.56×10^{-2}	2.86×10^{-1}	-1.54
Carbon metabolism_Homo sapiens_hsa01200	1.1×10^{-2}	2.34×10^{-1}	-1.40
Chronic myeloid leukemia_Homo sapiens_hsa05220	2.09×10^{-2}	3.57×10^{-2}	-1.44
Ubiquitin mediated proteolysis_Homo sapiens_hsa04120	1.54×10^{-2}	2.86×10^{-2}	-1.33
Oocyte meiosis_Homo sapiens_hsa04114	2.34×10^{-2}	3.57×10^{-2}	-1.43
Hippo signaling pathway_Homo sapiens_hsa04390	2.25×10^{-2}	3.57×10^{-2}	-1.35
Pyrimidine metabolism_Homo sapiens_hsa00240	2.69×10^{-2}	3.9×10^{-1}	-1.29
FoxO signaling pathway_Homo sapiens_hsa04068	4.4×10^{-2}	4.76×10^{-1}	-1.29

HumanCyc 2016 via Enrichr			
Term	P-value	Adjusted p-value	Z score
superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis_Homo sapiens_PWY-7211	3.76×10^{-3}	1.65×10^{-2}	-2.04
superpathway of conversion of glucose to acetyl CoA and entry into the TCA cycle_Homo sapiens_PWY66-407	68.4×10^{-4}	1.65×10^{-2}	-1.50
tRNA charging_Homo sapiens_TRNA-CHARGING-PWY	6.7×10^{-3}	1.65×10^{-2}	-1.45
glycolysis_Homo sapiens_PWY66-400	7.68×10^{-3}	1.65×10^{-2}	-1.15
UTP and CTP de novo biosynthesis_Homo sapiens_PWY-7176	9.9×10^{-3}	1.7×10^{-2}	-1.18
superpathway of pyrimidine ribonucleotides de novo biosynthesis_Homo sapiens_PWY0-162	17.7×10^{-3}	2.45×10^{-1}	-1.13
pyrimidine deoxyribonucleotides biosynthesis from CTP_Homo sapiens_PWY-7210	22.7×10^{-3}	2.45×10^{-1}	-1.03
pyrimidine deoxyribonucleotides de novo biosynthesis_Homo sapiens_PWY-7184	22.7×10^{-3}	2.45×10^{-1}	-0.82
superpathway of pyrimidine deoxyribonucleoside salvage_Homo sapiens_PWY-7200	28.5×10^{-3}	2.73×10^{-1}	-0.86
superpathway of purine nucleotide salvage_Homo sapiens_PWY66-409	5.1×10^{-2}	3.13×10^{-2}	-0.04
superpathway of tryptophan utilization_Homo sapiens_PWY66-401	9.5×10^{-1}	9.5×10^{-1}	10.9
mucin core 1 and core 2 O-glycosylation_Homo sapiens_PWY-7433	8.9×10^{-1}	9×10^{-1}	13.1
3-phosphoinositide biosynthesis_Homo sapiens_PWY-6352	8.8×10^{-1}	9×10^{-1}	12.3
adenosine ribonucleotides de novo biosynthesis_Homo sapiens_PWY-7219	8.6×10^{-1}	9×10^{-1}	11.5
D-myo-inositol (1,4,5)-trisphosphate biosynthesis_Homo sapiens_PWY-6351	8.6×10^{-1}	9×10^{-1}	12.1
glutathione-mediated detoxification_Homo sapiens_PWY-4061	8.5×10^{-1}	9×10^{-1}	11.7
guanosine nucleotides de novo biosynthesis_Homo sapiens_PWY-7228	10×10^{-2}	4.46×10^{-1}	0.99
phospholipases_Homo sapiens_LIPASYN-PWY	7.94×10^{-1}	8.53×10^{-1}	11.2
superpathway of inositol phosphate compounds_Homo sapiens_PWY-6371	7.67×10^{-1}	8.45x10-1	10.3
3-phosphoinositide degradation_Homo sapiens_PWY-6368	7.93×10^{-1}	8.53x10-1	13.7

BioCarta 2016 via Enrichr			
Term	P-value	Adjusted p-value	Z score
Eukaryotic protein translation_Homo sapiens_h_eifPathway	$30.1x10^{-6}$	$5.42x10^{-3}$	-1.34
Caspase Cascade in Apoptosis_Homo sapiens_h_caspasePathway	$6.15x10^{-3}$	$2.76x10^{-1}$	-1.37
BTG family proteins and cell cycle regulation_Homo sapiens_h_btg2Pathway	$3.03x10^{-3}$	$2.72x10^{-1}$	-0.98
Cell Cycle: G1/S Check Point _Homo sapiens_h_g1Pathway	$11.5x10^{-3}$	$2.96x10^{-2}$	-1.08
CDK Regulation of DNA Replication_Homo sapiens_h_mcmPathway	$9.22x10^{-3}$	$2.76x10^{-2}$	-1.01
Internal Ribosome entry pathway_Homo sapiens_h_iresPathway	$9.22x10^{-3}$	$2.76x10^{-2}$	-0.94
HIV-1 Nef: negative effector of Fas and TNF_Homo sapiens_h_HivnefPathway	$17.1x10^{-3}$	$4x10^{-1}$	-1.05
Cell Cycle: G2/M Checkpoint_Homo sapiens_h_g2Pathway	$2.2x10^{-2}$	$4x10^{-1}$	-0.91
Apoptotic DNA fragmentation and tissue homeostasis_Homo sapiens_h_DNAfragmentPathway	$7x10^{-3}$	$3x10^{-1}$	-0.52
Influence of Ras and Rho proteins on G1 to S Transition_Homo sapiens_h_RacCycDPathway	$5.65x10^{-2}$	$5.47x10^{-2}$	-0.89
Telomeres, Telomerase, Cellular Aging, and Immortality_Homo sapiens_h_telPathway	$2.27x10^{-2}$	$3.95x10^{-2}$	-0.59
Cyclins and Cell Cycle Regulation_Homo sapiens_h_cellcyclePathway	$26.4x10^{-3}$	$3.95x10^{-2}$	-0.47
IGF-1 Signaling Pathway_Homo sapiens_h_igf1Pathway	$6x10^{-2}$	$5.47x10^{-1}$	-0.35
Chaperones modulate interferon Signaling Pathway_Homo sapiens_h_tidPathway	$4.26x10^{-2}$	$5.1x10^{-1}$	-0.20
ATM Signaling Pathway_Homo sapiens_h_atmPathway	$4.26x10^{-2}$	$5.1x10^{-1}$	-0.10
Phospholipids as signalling intermediaries_Homo sapiens_h_edg1Pathway	$10x10^{-2}$	$5.65x10^{-1}$	-0.06
Actions of Nitric Oxide in the Heart_Homo sapiens_h_no1Pathway	$9.63x10^{-1}$	$9.63x10^{-1}$	7.53
Ceramide Signaling Pathway_Homo sapiens_h_ceramidePathway	$10x10^{-2}$	$5.65x10^{-1}$	0.17
Trefoil Factors Initiate Mucosal Healing_Homo sapiens_h_tffPathway	$13.3x10^{-2}$	$6.15x10^{-1}$	0.24
Chromatin Remodeling by hSWI/SNF ATP-dependent Complexes_Homo sapiens_h_hSWI-SNFpathway	$2.85x10^{-2}$	$4x10^{-1}$	0.16

Reactome 2016 via Enrichr			
Term	P-value	Adjusted p-value	Z score
Gene Expression_Homo sapiens_R-HSA-74160	1.73×10^{-22}	2.06×10^{-19}	-2.15
Infectious disease_Homo sapiens_R-HSA-5663205	2×10^{-13}	2×10^{-10}	-2.39
Cell Cycle, Mitotic_Homo sapiens_R-HSA-69278	14.8×10^{-12}	5.9×10^{-9}	-2.45
Cell Cycle_Homo sapiens_R-HSA-1640170	6.8×10^{-11}	1.62×10^{-9}	-2.42
Translation_Homo sapiens_R-HSA-72766	2.67×10^{-11}	8×10^{-9}	-2.01
Disease_Homo sapiens_R-HSA-1643685	12.4×10^{-9}	1.23×10^{-7}	-2.29
Cap-dependent Translation Initiation_Homo sapiens_R-HSA-72737	10.2×10^{-10}	1.73×10^{-8}	-1.94
Eukaryotic Translation Initiation_Homo sapiens_R-HSA-72613	10.2×10^{-10}	1.73×10^{-8}	-1.93
Influenza Infection_Homo sapiens_R-HSA-168254	3.2×10^{-9}	4.74×10^{-7}	-2.02
Cell Cycle Checkpoints_Homo sapiens_R-HSA-69620	2.7×10^{-8}	1.85×10^{-7}	-2.22
Influenza Life Cycle_Homo sapiens_R-HSA-168255	5.87×10^{-9}	7×10^{-7}	-2.01
GTP hydrolysis and joining of the 60S ribosomal subunit_Homo sapiens_R-HSA-72706	4.2×10^{-9}	5.53×10^{-7}	-1.95
Influenza Viral RNA Transcription and Replication_Homo sapiens_R-HSA-168273	1.9×10^{-8}	1.52×10^{-7}	-1.96
Peptide chain elongation_Homo sapiens_R-HSA-156902	8.1×10^{-9}	8.75×10^{-7}	-1.87
mRNA Splicing - Major Pathway_Homo sapiens_R-HSA-72163	1.55×10^{-8}	1.42×10^{-7}	-1.89
Cellular responses to stress_Homo sapiens_R-HSA-2262752	1.64×10^{-7}	7.51×10^{-6}	-2.15
mRNA Splicing_Homo sapiens_R-HSA-72172	2.56×10^{-8}	1.85×10^{-6}	-1.89
Eukaryotic Translation Elongation_Homo sapiens_R-HSA-156842	2.8×10^{-8}	1.85×10^{-6}	-1.86
Eukaryotic Translation Termination_Homo sapiens_R-HSA-72764	1.72×10^{-8}	1.47×10^{-6}	-1.81
Processing of Capped Intron-Containing Pre-mRNA_Homo sapiens_R-HSA-72203	1.32×10^{-7}	6.31×10^{-6}	-1.96

WikiPathways 2016 via Enrichr			
Term	P-value	Adjusted p-value	Z score
mRNA processing_Mus musculus_WP310	6.75×10^{-11}	2.48×10^{-8}	-2.13
mRNA Processing_Homo sapiens_WP411	6.07×10^{-8}	1.11×10^{-6}	-1.94
Cytoplasmic Ribosomal Proteins_Homo sapiens_WP477	5.5×10^{-7}	6.75×10^{-5}	-1.95
Cytoplasmic Ribosomal Proteins_Mus musculus_WP163	13.4×10^{-6}	1.24×10^{-4}	-1.94
Retinoblastoma (RB) in Cancer_Homo sapiens_WP2446	3.62×10^{-5}	2.67×10^{-3}	-1.84
XPodNet - protein-protein interactions in the podocyte expanded by STRING_Mus musculus_WP2309	13.7×10^{-5}	5.62×10^{-3}	-2.01
miRNA regulation of DNA Damage Response_Mus musculus_WP2087	6.67×10^{-5}	4.1×10^{-3}	-1.83
Translation Factors_Homo sapiens_WP107	8.01×10^{-5}	4.21×10^{-3}	-1.85
Translation Factors_Mus musculus_WP307	11.7×10^{-5}	5.41×10^{-3}	-1.70
PluriNetWork_Mus musculus_WP1763	4.4×10^{-4}	1.35×10^{-3}	-1.89
Cell Cycle_Homo sapiens_WP179	2.41×10^{-4}	8.9×10^{-3}	-1.66
Androgen receptor signaling pathway_Homo sapiens_WP138	3.28×10^{-4}	1.1×10^{-2}	-1.63
DNA Damage Response_Homo sapiens_WP707	4.89×10^{-4}	1.38×10^{-3}	-1.69
PodNet: protein-protein interactions in the podocyte_Mus musculus_WP2310	9.09×10^{-4}	2.4×10^{-2}	-1.81
Delta-Notch Signaling Pathway_Mus musculus_WP265	1×10^{-3}	2.47×10^{-2}	-1.59
G1 to S cell cycle control_Mus musculus_WP413	1.57×10^{-3}	3.63×10^{-2}	-1.54
Circadian rythm related genes_Homo sapiens_WP3594	2.43×10^{-3}	3.86×10^{-2}	-1.65
Integrated Pancreatic Cancer Pathway_Homo sapiens_WP2377	2.77×10^{-3}	3.86×10^{-2}	-1.62
miRNA Regulation of DNA Damage Response_Homo sapiens_WP1530	2.12×10^{-3}	3.86×10^{-2}	-1.52
Proteasome Degradation_Homo sapiens_WP183	2.11×10^{-3}	3.86×10^{-2}	-1.42

NCI-Nature 2016 via Enrichr			
Term	P-value	Adjusted p-value	Z score
Regulation of Telomerase_Homo sapiens_4dfe97ca-6195-11e5-8ac5-06603eb7f303	$3x10^{-5}$	$2.84x10^{-4}$	-1.64
ATR signaling pathway_Homo sapiens_8991cbac-618b-11e5-8ac5-06603eb7f303	$1.94x10^{-5}$	$2.84x10^{-4}$	-1.43
p53 pathway_Homo sapiens_a0de862d-6194-11e5-8ac5-06603eb7f303	$6.69x10^{-5}$	$4.22x10^{-3}$	-1.54
Aurora B signaling_Homo sapiens_304a75af-618c-11e5-8ac5-06603eb7f303	$1.04x10^{-4}$	$4.94x10^{-3}$	-1.19
Coregulation of Androgen receptor activity_Homo sapiens_27e0e369-6191-11e5-8ac5-06603eb7f303	$3.86x10^{-4}$	$1.21x10^{-3}$	-1.32
ATM pathway_Homo sapiens_49bc3e2b-618b-11e5-8ac5-06603eb7f303	$1.46x10^{-4}$	$5.54x10^{-3}$	-1.16
mTOR signaling pathway_Homo sapiens_559dd850-6194-11e5-8ac5-06603eb7f303	$5.72x10^{-4}$	$1.35x10^{-3}$	-1.37
E2F transcription factor network_Homo sapiens_bb4d0fd3-6191-11e5-8ac5-06603eb7f303	$8.93x10^{-4}$	$1.53x10^{-3}$	-1.30
Validated targets of C-MYC transcriptional activation_Homo sapiens_61d3b115-6196-11e5-8ac5-06603eb7f303	$7.71x10^{-4}$	$1.45x10^{-3}$	-1.22
Notch-mediated HES/HEY network_Homo sapiens_8ee56389-6194-11e5-8ac5-06603eb7f303	$7.51x10^{-4}$	$1.45x10^{-3}$	-1.12
Notch signaling pathway_Homo sapiens_88f83518-6194-11e5-8ac5-06603eb7f303	$1.35x10^{-3}$	$2.13x10^{-2}$	-1.04
Regulation of retinoblastoma protein_Homo sapiens_407a3468-6195-11e5-8ac5-06603eb7f303	$2.8x10^{-3}$	$3.78x10^{-2}$	-1.10
TGF-beta receptor signaling_Homo sapiens_1f188fcc-6196-11e5-8ac5-06603eb7f303	$6.7x10^{-3}$	$8.44x10^{-2}$	-0.93
Regulation of nuclear SMAD2/3 signaling_Homo sapiens_246aac04-6195-11e5-8ac5-06603eb7f303	$7.6x10^{-3}$	$8.5x10^{-2}$	-0.94
Caspase Cascade in Apoptosis_Homo sapiens_b9d3ef2e-618f-11e5-8ac5-06603eb7f303	$7.64x10^{-3}$	$8.5x10^{-2}$	-0.90
Aurora A signaling_Homo sapiens_f131cf8e-618b-11e5-8ac5-06603eb7f303	$1.73x10^{-3}$	$2.52x10^{-2}$	-0.57
Validated nuclear estrogen receptor alpha network_Homo sapiens_58949883-6196-11e5-8ac5-06603eb7f303	$2.14x10^{-2}$	$1.92x10^{-1}$	-0.70
Trk receptor signaling mediated by PI3K and PLC-gamma_Homo sapiens_4037def0-6196-11e5-8ac5-06603eb7f303	$16.8x10^{-3}$	$1.67x10^{-2}$	-0.55
Circadian rhythm pathway_Homo sapiens_ffb11b85-6190-11e5-8ac5-06603eb7f303	$5.15x10^{-4}$	$1.35x10^{-3}$	-0.27
p75(NTR)-mediated signaling_Homo sapiens_b492782f-6194-11e5-8ac5-06603eb7f303	$3.13x10^{-2}$	$2.04x10^{-2}$	-0.49

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